The Interferon alpha receptor utilises T-cell receptor-associated proteins for signalling

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ABSTRACT

The interferon alpha receptor (IFNAR) and T-cell receptor (TCR) are expressed upon the T-cell surface. The dimeric Class I interferon receptor is a cytokine receptor that recognises interferons such as IFNα. Interferons (IFNs) are pluripotent, antiviral cytokines that causes antiproliferative effects, primarily through Jak/STAT signalling. The T-cell receptor is an antigenic receptor that recognises antigenically-derived peptides in the context of the MHC complex located on an antigen presenting cell, resulting in a cellular proliferation. Although both receptors elicit opposing cellular outcomes, both the TCR and IFNAR activate the ERK MAPK signalling pathway, albeit with a different time course. Furthermore, studies have shown that the IFNAR and TCR utilise an overlapping subset of proteins for this pathway to occur such as CD45, Lck and Zap70.

In this study evidence is presented to show that two further TCR-associated proteins are phosphorylated in response to the IFNAR; the 95kDa guanosine nucleotide exchange factor, Vav, and the 76kDa adaptor protein Slp76. This proceeds in a similar manner to that observed at the TCR. Furthermore, the absence of either protein impairs IFNAR-induced ERK MAPK signalling. The similarities between TCR and IFNAR signalling led to questioning of whether crosstalk occurs between the two receptors. To address this possibility a TCRβ deficient cell line, which lacks functional TCR expression, was utilised. It was demonstrated that the absence of the TCR completely abrogates the ERK MAPK response emanating from the IFNAR yet Jak/STAT signalling is unaffected. These results highlight for the first time an intimate connection between the TCR and IFNAR.

DECLARATION

"I, Miss Claire Naomi Stevens, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis."

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Claire N. Stevens

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CONTENTS

FIGURES

CHAPTER 4: A Newly defined role for Slp76 in Interferon Alpha Receptor Signalling

CHAPTER 5: Investigating cross-talk between the TCR and IFNAR

ABBREVIATIONS

ADAP: Adhesion and degranulation-promoting adaptor protein

ATP: Adenosine triphosphate

AP-1: Activator protein 1

α-AR: Alpha adrenergic receptor

APC: Antigen-presenting cell

BCR: B-cell receptor

β-AR: Beta adrenergic receptor

BMK: Big Mitogen-activated protein kinase

CRAC: Calcium release-activated calcium channel

DAG: Diacylglycerol

ddH_2O: Distilled and autoclaved water

DH: Dbl Homology

EGF: Epidermal growth factor

ER: Endoplasmic Reticulum

ERK: Extracellular signal-regulated kinase

FRET: Fluorescence resonance energy transfer

Gads: Grb2- related adaptor downstream of Shc

GEF: Guanisine nucleotide exchange factor

GDP: Guanisine di-phosphate

GPCR: G-protein coupled receptor

Grb2: Growth factor receptor bound protein

GFP: Green fluorescent protein

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GTP: Guanisine tri-phosphate

HPK-1: Hematopoietic progenitor kinase 1

IFNα or IFNγ: Interferon Alpha or Interferon Gamma

IL-2/IL-2R: Interleukin-2/Interleukin-2 receptor

IκB: Inhibitory κB protein

IFNAR: Interferon alpha receptor

IGF-1: Insulin-like growth factor

IP3: Inositol triphosphate

ISG: Interferon-stimulated gene

ISRE: Interferon-stimulated response element

Itk: Inducible T-cell kinase

ITAM: Immunoreceptor Tyrosine-based Activation Motif

ITIM: Immunoreceptor tyrosine-based inhibition motif

JNK: Jun N-terminal kinase

LAT: Linker for activated T-cells

MAPK: Mitogen-activated protein kinase

MAPKK or MAP2K: Mitogen-activated protein kinase kinase

MAPKKK or MAP3K: Mitogen-activated protein kinase kinase kinase

MAP4K: Mitogen-activated protein kinase kinase kinase kinase

MHC: Major histocompatibility complex

NES: Nuclear entry signal

NFAT: Nuclear factor

NFκB: Nuclear Factor

NGF: Nerve growth factor

NRTK: Non-receptor tyrosine kinase

OD: Optical density

PAK: p21/Cdc42/Rac1-activated kinase 1

PH: Pleckstrin Homology

PI3K: Phosphadityl inositol 3 kinase

PIP2: Phosphatidylinositol (4,5) bisphosphate

PIP3: Phophatidylinositol (3,4,5) triphosphate

PKA: Protein kinase A

PKC: Protein kinase B

PLCβ: Phospholipase C Beta

PKCθ: Protein kinase C theta

PLC: Phospholipase C

PTB: Phospho-tyrosine binding

PTK: Protein tyrosine kinase

PTPs: Protein tyrosine phosphatases

pTyr or pY: Phosphotyrosine

RFP: Red fluorescent protein

RPM: Rotations per minute

RTK: Receptor tyrosine kinase

S1P: Sphingosine 1 phosphate

SAM: Sterile alpha motif

Ser: Serine

SH2/SH3: Src homology type 2 or 3

SKAP-55: SRC kinase associated phosphoprotein 55KD

Slp65: Src homology 2 domain containing leukocyte protein of 65 kDa

Slp76: Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa

Sos: Son of Sevenless

STAT: Signal transducer and transactivator

TIRF: Total internal reflection microscopy flourescence

TCR: T-cell receptor

Thr: Threonine

TNF-α: Tumour-necrosis factor-α

Tyr or Y: tyrosine

UN: Unique region

WASP: Wiscott-Aldrich syndrome protein

Zap70: ζ Chain-associated protein kinase of 70kd

CHAPTER 1

Introduction

1.1. Spatio-temporal control of signalling complexes within the cell

The cell is continuously receiving information from its extracellular environment regarding how it should behave. It is extremely well adapted to be able to decipher the stimuli it has received and thus react according. Instructions communicated to the cell come mainly in the form of peptide ligands. These ligands are then able to bind to their cognate receptors, located on the cell surface, and instigate changes intracellularly such as phosphorylation on tyrosine or serine residues. Initial phosphorylation can occur through autophosphorylation, in the case of receptor tyrosine kinases, or through the enzymatic activity of associated protein tyrosine kinases. Upon peptide recognition, a ligated receptor must transmit the signal it has obtained at the cell surface towards the nucleus of the cell where the appropriate gene transcription will ensue causing a unique effector response to be elicited such as growth, differentiation, survival, apoptosis and morphological changes. The precise response is reliant on which receptor has been stimulated. The mode in which information is conveyed through the cell is via carefully orchestrated multi-protein signalling cascades involving specific subsets of proteins being sequentially or simultaneously activated through interactions with different binding partners. There are two possible explanations as to how these pathways are perpetuated, linearly or via multi-molecular interactions.

1.1.1 Linear Signalling

Signalling cascades have been traditionally represented as linear type cascades involving a series of bi-molecular interactions where one activated protein then activates the next protein in the chain and so on until a point is reached where an active protein translocates to the nucleus to bind to a gene promoter to evoke transcription. Proteins involved in such receptor-mediated signalling consist of characteristic interaction domains and motifs that direct their constitutive or signal-regulated association with specific binding partners. Protein-protein interaction domains include the compact 100 amino acid SH2 domain, which recognises phosphotyrosine motifs, the 60 amino acid SH3 domain, which recognises proline-rich regions and PTB, which recognises a different phosphotyrosine motif to the SH2 domain. All of these interaction domains, as well as other defined domains, share a distinctive structure and are highly homologous in much of their sequence thus allowing each domain to be categorised. There are over 110 SH2 domains and nearly 300 SH3 domains (Pawson, 2002). Another common domain that mediates protein-lipid interactions and is particularly prevalent in proteins recruited to membrane-bound receptors, is the Pleckstrin Homology (PH) domain of which there are over 200 (Pawson, 2002). Other common domains include the WW, FYVE, PDZ domains. Although these domains display a degree of specificity, their binding is actually quite promiscuous because their similarities and their binding affinities are not strong enough to stop "wrong" interactions occurring (O'Rourke & Ladbury, 2003). Given that a cell expresses a plethora of proteins encompassing one or more of these characteristic binding modules, incorrect contacts would become a likelihood and could result in disruption of protein pathways culminating in incorrect gene transcription if this situation arose. Therefore, linear

signalling does not attribute a high enough magnitude of control over signalling events and investigations have shown that a series of complex interactions are instead required for mutually exclusive signalling as discussed in the following section.

1.1.2 Multi-molecular Signalling

A much more likely scenario of how a receptor is able to ensure integrity of exact signalling pathways is through the contemporaneous, multivalent binding of proteins immediately post-receptor ligation to form an early signalling complex at the receptor. The concurrent binding of proteins to one another through their multiple domains would allow more precise interactions to be achieved and allow more stable interactions to be generated (See Fig. 1.1).

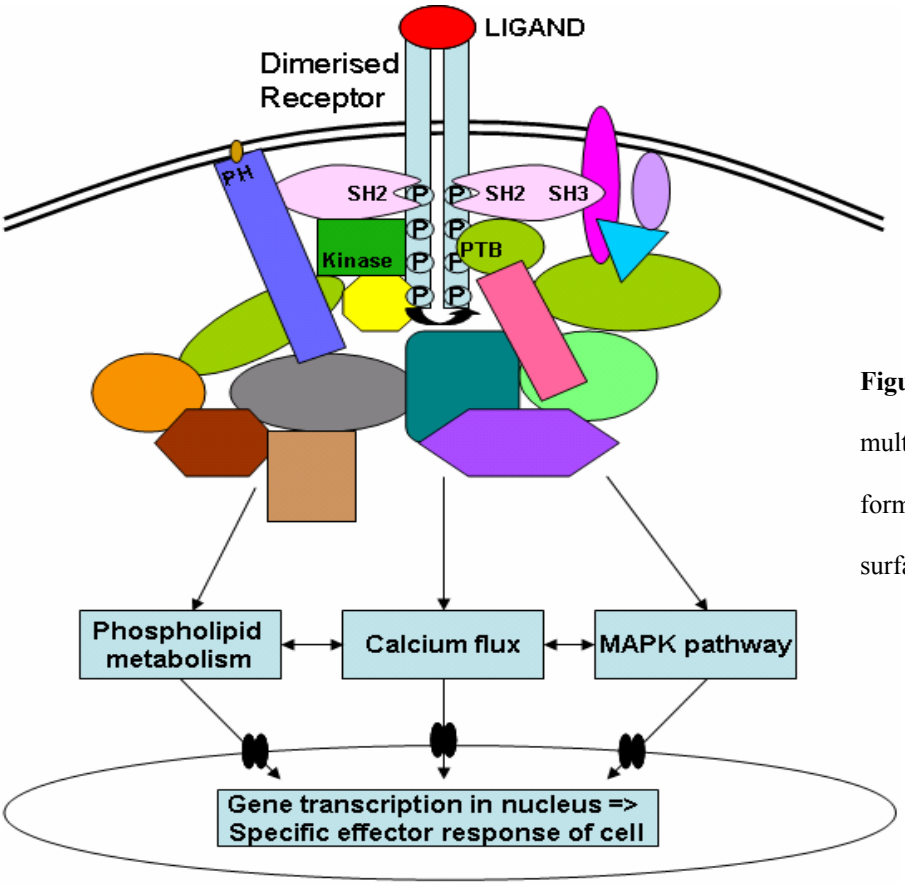


Figure 1.1 Cartoon of how multimolecular complexes form at phosphorylated cell surface receptors e.g. RTKs

There are thousands of different cell surface receptors upon each cell and these are constantly being roused and are responsible for triggering a myriad of proteins to engage in signalling cascades that ultimately control the growth, survival, cell cycle, differentiation, size, and eventually death of each cell. There are many questions that arise when considering how receptor-mediated events remain mutually exclusive. Many proteins can be activated by more than one single receptor so one important question to ask is how does a single protein "know" what receptor it has been stimulated in response to and thus what other proteins it should subsequently bind to and/or activate? Many proteins are able to bind one or more common partners at alternative receptors within the same cell so clearly there must be a meticulous mechanism to stop false associations or incorrect pathways being activated at these differing receptors. Another important issue to address is how specificity is maintained between receptors if many cell surface receptors can activate overlapping groups of proteins and trigger many common signalling cascades downstream e.g. phospholipid metabolism, MAPK activation and changes in ion concentrations such as calcium.

The formation of multimolecular complexes at the receptor attributes a manner in which specificity is achievable and is now recognised that this is an accurate representation of the events that occur at most receptors. Each component is recruited into a multimeric complex formed at the receptor at a particular time point and will leave the complex at a distinct time point also. The length of time that a given protein spends at the signalling complex, bound to a collection of proteins will vary depending on the receptor that has been provoked. The spatial arrangement and composition of the complex will alter over time and

will vary when drawing comparisons between receptors. The rigid signalling mechanisms at a receptor will ultimately control signalling events downstream and bring about the appropriate response. Specific examples of receptors that utilise overlapping sets of proteins whilst still maintaining exclusive responses are discussed in the remainder of this introduction. The modes in which the information is carried to the nucleus e.g. through tyrosine phosphorylation, which triggers ERK MAPK signalling is also discussed.

1.2. Utilisation of tyrosine phosphorylation for cell to cell communication

Receptors were first discovered 35 years ago through applying insulin, EGF and NGF (Gshwind et al., 2004). They are usually membrane spanning proteins with a ligand-binding extracellular domain, a transmembrane domain and an intracellular domain. Proteins recruited intracellularly need to be able to transmit the information, initially received in the form of a ligand binding to the extracellular domain of the receptor, from the cell surface to the nucleus in order to bring about the correct outcome according to the signal received. The information is carried through a series of activation-dependent post-translational modifications to intracellular proteins. One such modification is achieved through tyrosine phosphorylation, which involves the transfer of the γ phosphate of ATP to hydroxyl groups of tyrosines on target proteins. This process is catalysed by protein tyrosine kinase enzymes. The addition of a phosphate alters the biophysical character of the residue because of the addition of a bulky, negatively charged group thus inducing conformational changes that increase the catalytic activity of the enzyme. Phosphate

addition may stabilise the interaction between the protein containing the phospho-tyrosine motif and domains on a binding partner e.g. SH2 or PTB domains (Wange, 2000). Receptors that rely upon tyrosine phosphorylation contain tyrosine motifs within the cytoplasmic region. The purpose of phosphorylating these residues is to create docking sites, which can then subsequently recruit other proteins and initiate further phopshorylation events.

Some receptors contain a catalytic kinase domain able to phosphorylate tyrosine residues upon a neighbouring receptor chain. This kinase activity is only activated upon ligation and is imperative for the initial tyrosine phosphorylation that takes place at the receptor. These auto-catalytic receptors do not require any additional kinases for the initial phosphorylation due to the ability to autophosphorylate within a receptor dimer. These receptors are termed receptor tyrosine kinases (RTKs). Other receptors contain intracellular tyrosine motifs upon the intracellular portion but do possess any catalytic kinase activity and instead rely on other cytoplasmic proteins to perform the role of tyrosine phosphorylation. These kinases are termed non-receptor tyrosine kinases (NRTKs).

1.2.1 Receptor Tyrosine Kinases

This class of receptors possesses intrinsic catalytic protein tyrosine kinase ability. There are over 60 types of RTK, belonging to 20 families. The largest mammalian family of RTKs are the Ephrin Receptor subfamily (Pawson, 2002). All RTKs comprise three main regions. The first is a large glycosylated extracellular domain, which typically contains a range of discrete globular domains such as the immunoglobulin (Ig)-like domains, fibronectin type III domains, cysteine-rich domain, and EGF-like domains (Hubbard & Till, 2000). Each

RTK also has transmembrane portion, composed of a single spanning helix domain, and cytoplasmic portion containing a protein tyrosine kinase domain and multiple tyrosine residues (Aaronson, 2005). RTKs exist as monomers in the cell membrane with the exception of the Insulin Receptor family and the Met family, which exist as preformed dimers. Upon ligation, the monomeric RTKs dimerise through various mechanisms (Schlessinger, 2000). Ligand-induced dimerisation results in activation of the receptor through trans-phosphorylation between the two receptor chains. This leads to phosphorylation of tyrosine residues within the activation loop on the intracellular chain of each receptor sub-unit through kinase activity of the other subunit. The phosphotyrosines then act as docking sites for SH2 and PTB-domain containing enzymes, adaptor and docking proteins, which serve to further nucleate complex formation and also initiate downstream signalling through further rounds tyrosine and serine/threonine phosphorylation. Down-regulation of RTK signalling occurs in via several processes such as receptor-mediated endocytosis, ubiquitin-directed proteolysis, and the action of protein tyrosine phosphatases (Hubbard &Till, 2000).

1.2.2 Non-Receptor Tyrosine Kinases

Some receptors lack intrinsic tyrosine kinase activity and instead rely upon constitutively associated NRTKs to phosphorylate specific tyrosine residues upon the cytoplasmic tail of the receptor. NRTKs are cytoplasmic proteins, but may be anchored to the membrane through palmitoylation or myristoylation. In addition to their kinase activity, they possess domains that mediate protein-protein, protein-lipid and protein-DNA interactions. The largest family of NRTKs are the Src family of which there are nine members including

Lck, which is required for T-cell receptor signalling and Lyn, required for B-cell receptor signalling. The other Src kinases are Hck, Blk, Fyn, Fgf, Yes and Yrc. All of the family share a similar structure; a membrane targeted N-terminal region, which is always myristoylated and sometimes palmitoylated, followed by an SH3 domain, an SH2 domain and a kinase domain in the C-terminus (Boggon & Eck, 2004). Another group of NRTKs are the Syk family of which Zap70, an essential T-cell receptor kinase, is a member. A further important family of NRTKs are the JAK family, which were originally found to be required for interferon signalling but are now known to be constitutively associated with the entire cytokine receptors family. The phosphotyrosines created by JAKs can then act as docking sites for STAT proteins and consequently JAK/STAT signalling commences, which is discussed in detail in section. The last two NRTK families are called Abl kinases and the other family is called Tec kinases, which includes Itk, an important protein in the signalling complex formed at the T-cell receptor.

Both the B-cell receptor (BCR) and the T-cell receptor (TCR), required for recognition of specific antigenic epitopes as part of the adaptive immune response, rely upon NRTKs for intracellular phosphorylation. These dimeric, clonotypic receptors lack an intracellular signalling domain, but instead are non-covalently linked to intracellular signalling chains that contain the tyrosine motifs acted upon by the NRTKs e.g. Lck in the case of the TCR and Lyn in the case of the BCR.

As with RTKs, down-regulation mechanisms are also needed in order to terminate signalling for NRTKs. This is achieved through an intimate relationship with phosphatases such as SHP-1 and CD45, receptor endocytosis and ubiquitination (aided by ubiquitin ligases such as Cbl). Also, both NRTKs have been found to mediate crosstalk with GPCRs

and Src family kinases seem to be especially important in linking GPCRs with the ERK signalling module. This is discussed in the next section and is reviewed in Luttrell & Luttrell, 2004.

1.2.3 Cross-talk between GPCRs and RTKs/NRTKs

The genes for GPCRs account for 5% of the human genome encoding over 1000 GPCRs, making this the largest class of receptors expressed upon the cell surface (Gavi et al., 2006) They are receptors that have a transmembrane region spanning the membrane seven times and they recognise a range of ligands including neurotransmitters, hormones, phospholipids, odorants and photons, in the case of phototransduction in the eye. Sometimes a specific ligand can also activate multiple members of the GPCR family also (Gavi et al., 2006). The cytoplasmic domain of the GPCR is associated with one or more heterotrimeric G proteins, composed of α, β and γ subunits. When the GPCR is activated, the exchange of bound GDP for GTP is stimulated by the α subunit, which then dissociates from the βγ subunit. These two units then stimulate the activities of enzymatic effectors such as adenylate cyclases, PLCβ isoforms and ion channels, subsequently leading to second messenger products such as PKA and PKC, which in turn trigger signalling cascades. A large body of evidence now illustrates that GPCRs can not only activate their own specific signalling pathways, but can also activate RTKs or NRTKs in the absence of any growth factor. Notably, this confers numerous GPCRs with the ability to promote growth by linking them with the ERK MAPK signalling pathway and also, similar to RTKs such as EGFR, GPCRs can still access ERK via clathrin-coated pits. The reverse is also true, RTKs/NRTKs can utilise GPCR-coupled machinery to elicit a response. This

phenomenon has been labelled "transactivation" or "crosstalk" and typically defined as the share of information between receptors in order to modulate the signal. Crosstalk was first identified in the 1980s to explain some of the inconsistencies of agonists such as α-AR and β-AR (Gavi et al., 2006). In addition, it has now emerged that some RTKs can transactivate other RTKs. Insulin-like growth factor-1 (IGF-1) can activate the EGFR, which results in ERK phosphorylation and phosphorylation of BAD protein, an anti-apoptotic Bcl-2 family member. There are four main ways in which GPCRs activate other receptors. The first involves "inside out" signalling whereby a GPCR transactivates an RTK family receptor through release of a soluble ligand that acts in an autocrine manner back on the cell e.g. EGF or PDGF receptor (discussed in example 2 below). A second mode of GPCR/RTK crosstalk involves a GPCR modulating cell surface expression of an RTK such as that observed between the Insulin RTK and the β-adrenergic GPCR (β-AR), which in concert to regulate glucose metabolism (See example 1 below). The third manner in which crosstalk takes place occurs between GPCRs and integrin receptors in adherent cells (see example 3). Finally RTKs can often act to activate GPCRs (see example 4).

1.2.3.1 Examples of cross-talk between RTKs and GPCRs:-

(1) The RTKs Insulin and IGF-1 influences the β_2AR (β_2 -adrenergic receptor) GPCR in glucose metabolism

Insulin promotes glycogen synthesis, glucose uptake in muscle and lipid storage whereas catecholamines exert an opposing effect by stimulating the breakdown of glycogen, gluconeogenesis and lipolysis (Gavi et al., 2006). The crosstalk that occurs between the

β_2ARs and IGF-1 in the regulation of glucose metabolism has been well studied and occurs at several direct intersections. Insulin is able to trigger internalisation of β_2AR, which attenuates the action of catecholamines. Molecules that have shown to mediate this scenario are β-arrestin and Akt. There are many other instances of cross-talk involving β_2ARs such as within the cardiovascular system

(2) EGFR-GPCR Cross-talk

There are two models to describe GPCR activation of EGFR signalling at present. The first is the ligand INDEPENDENT mode of activation that employs factors such as PKC, Src and PI3K e.g. β_2ARs (Maudsley et al., 2000) and angiotensin receptors (Seta et al., 2003) have been shown to directly interact with the EGFR. The second model involves an 'inside-out' mechanism whereby a GPCR activates a metalloprotease, which results in ectodomain shedding i.e. the proteolytic cleavage of a membrane-bound EGFR pro-ligand, which once released can act back upon the same cell and bind to the EGFR. EGFR signal transduction then proceeds in a manner that is almost the same as when it is activated by its normal ligand (Werry et al., 2005). This mode is attributable to thrombin, endothelin, acetylcholine and lysophosphatic acid (LPA) receptors.

(3) GPCR-Integrin crosstalk

When integrin receptors form focal adhesions upon contact with the extracellular matrix, numerous signalling proteins are recruited to a signalling platform, which control cytoskeletal reorganisation and growth. Src family kinases can phosphorylate a protein called Focal Adhesion Kinase (FAK), which provides a platform for Grb2-SOS

recruitement and subsequent signalling through the ERK MAPK. A protein that shares 60% sequence identity with FAK is Pyk2. Pyk2 can perform a similar role to FAK and can be activated through the GPCR-induced release of intracellular calcium stores, which causes Pyk2 to autophosphorylate and recruit Grb2-SOS complexes at integrin receptors to trigger ERK MAPK signalling Therefore GPCRs can activate ERK MAPK signalling through Pyk2 at the integrin receptor (Luttrell & Lutrell, 2004).

(4) IGF-1 crosstalk with other GPCRs

IGF-1 activates the CCR5 chemokine receptor in MCF-7 cells via a transcriptional dependent mechanism. When IGF-1 is stimulated, the RANTES gene is upregulated and the product is secreted. This then acts in an autocrine manner to stimulate the CCR5 receptor and induce chemotaxis (Delcourt et al., 2006). IGF-1, NGF and PDGF can also activate the sphingosine 1 phosphate (S1P) receptor, which helps modulate cell growth e.g. neurite extension in the case of NGF, survival, differentiation and motility. Activation of the aforementioned RTKs can induce transcription of the S1P kinase (SphK), which subsequently activates the S1P receptor (El-Shewy et al, 2006, Hobson et al., 2001, Toman et al. 2004).

1.3 Pathways activated by RTKS and NRTKS

There are many signalling events catalysed by ligand-bound RTKs or by receptor-associated NRTKs. Two of the primary ones are the ERK MAPK signalling and phospholipid metabolism. In the following section, I shall discuss one of the best

characterised pathways emanating from growth factor receptors, the MAPK signalling pathway.

1.3.1 MAPK Signalling

The MAPK pathway is an ancient and evolutionarily conserved cascade that is responsible for transmitting signals received by receptors on the cell surface, such as RTKs, NRTKs, cytokines, GPCRs and integrins, to the transcriptional machinery in the nucleus. The MAPK pathways transduce signals that control a diverse range of fundamental cellular responses, which include proliferation and growth, differentiation, cell migration as well as stress-related responses such as cell cycle arrest and apoptosis. Mammalian MAPKs have been categorised into six groups based on phosphorylation consensus, sequence identity, signalling profile and functions (reviewed in Krens et al., 2006). These groups are:-

(i) Extra signal-regulated kinase (ERK) 1 and ERK2

(ii) ERK3 and ERK4

(iii) ERK5

(iv) ERK7 and ERK8

(v) Jun N-terminal kinases (JNK) 1, 2 and 3

(vi) p38 MAPK isoforms - α, β, γ (also known as ERK6) and δ

Similar signalling modules exist for activation of all the MAPKs. A MAP kinase kinase kinase (MAPKKK or MAP3K) is phosphorylated by a receptor-activated G-protein. Active MAPKKK in turn phosphorylates a MAP kinase kinase (MAPKK or MAP2K), which then

phosphorylates a MAP kinase (MAPK), which is the final link in the MAPK chain. MAPKs can then translocate to the nucleus to instigate transcriptional changes (see Fig. 1.2). The MAPKKK/MAPKK/MAPK signalling cascade has been remarkably conserved from plants, fungi, nematodes, insects to mammals (Widmann et al., 1999).

ERK1 and then ERK2 (also know as p44 and p42 respectively), were the first MAPKs to be identified in mammals (Rossomando et al., 1989) and are usually associated with cell growth in response to growth factors. In addition to ERK1 and 2, there are six other mammalian ERKs that have been identified; ERK3, 4, 5, 4, 7 and 8 (reviewed in Bogoyevitch et al., 2004), which are spread across the six groups of MAPKs. ERK3 was identified at the same time as ERK1/2. Since then, the other family members have been isolated, the most recent being ERK8. ERK5 is the largest ERK MAPK at 88kDa so is also known as Big MAPK (BMK). Relatively little is known about ERKs3-8 in comparison to ERK1/2 and much knowledge still needs to be accumulated regarding their functions, their downstream targets as well as how they are controlled through upstream signalling cascades and which receptors precisely activate them. Both the p38 MAPKs and JNK family are usually activated in response too cellular stress stimuli. In the research presented in the flowing chapters, the pathways leading to the phosphorylation of ERK1/2 are a main focus. This is described in much more depth in the following sections.

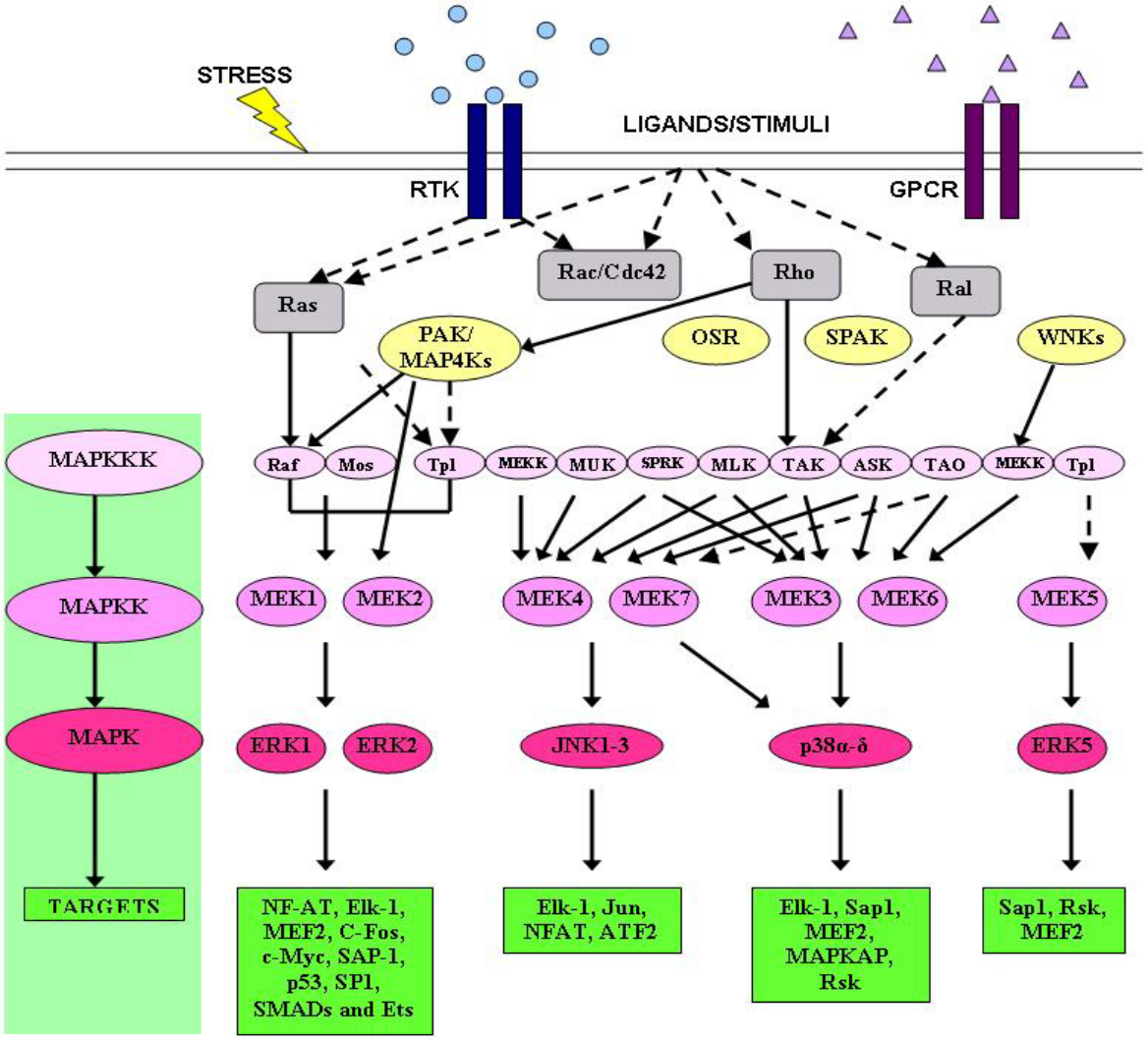


Figure 1.2 MAPK signalling induced by RTK (or GPCR) signalling. ***(Adapted from Raman et al., 2007).*** Cell surface receptor binding results in GTP loading onto small GTPases e.g. Ras. GTP bound GTPases subsequently activate MAPKKKs e.g. Raf. This then results in dual phosphorylation of a MAPKK such as MEK1/2, which lastly results in a MAPK family member being phosphorylated e.g. ERK1/2. This results in upregulation of a number of target genes in the nucleus

1.3.1.1 ERK1/2

The ERK1 and ERK2 enzymes are 43kDa and 41kDa respectively. They are ubiquitously expressed and share a high degree of similarity to one another; they are 75% similar at the amino acid level, which rises to 90% when the N-terminal stretch is omitted (Boulton et al.,

1990, Boulton et al., 1991). Despite their similarity, there does seem to be a certain degree of functional redundancy. ERK1 knockout mice are viable, fertile and of normal size and only thymocyte terminal differentiation is impaired. However, knocking out the ERK2 gene proves to be lethal in early embryonic stages (day 6.5), therefore ERK2 can compensate for some functions of ERK1 but not vice versa (Pages et al., 1999).

ERK1/2 are activated in response to cytokines and growth factors, phorbol esters, serum, GPCRs, transforming growth factors, osmotic stress and other cell stresses, and microtubule depolymerisation (Raman et al., 2007). The pathway that culminates in ERK phosphorylation is initiated by receptor ligation, except in cancerous cells. The ensuing auto-phosphorylation or phosphorylation by associated protein tyrosine kinases generates phosphotyrosines (pTyr) that act as docking sites for the SH2 domains of the adaptor protein, Grb2. The recruitment of Grb2 also brings the guanisine nucleotide exchange factor (GEF) Son-of-Sevenless (SOS) to the vicinity of the receptor as this protein is constitutively associated with Grb2 (Chardin et al., 1993).. Grb2 can also associate with the receptor through an indirect interaction whereby the adaptor Shc binds pTyr residues in the receptor through its PTB domain and then simultaneously binds to Grb2 thus tethering it to the receptor (Ravichandran, 1995). At the cell surface, the G-protein, Ras, is anchored to the membrane within lipid rafts encompassing the activated receptor. Once SOS is brought to the cell surface via Grb2, it catalyses the exchange of GTP for GDP bound to neighbouring Ras molecules. This allows Ras to interact with its target proteins such as the Raf family of kinases, which are a group of MAPKKKs, and comprises A-Raf, B-Raf and c-Raf (Leevers et al., 1994, Hangemann & Rapp, 1999). Rafs exist in an autoinhibited STATe in the cytosol and is stabilised through the interaction of 14-3-3 dimers with N- and

C-terminal phosphorylation sites. Association with activated Ras induces the release of bound 14-3-3 and relieves the autoinhibited STATe of Raf, allowing Ras access to the phosphorylation sites (Muslin et al., 1996, Thorson et al., 1998). Phosphorylation confers Raf with catalytic activity towards the next set of proteins in the MAPK signalling cascade, a group of MAPKKs called MEK proteins (Kyriakis et al., 1992). There are other families of MAPKKKs upstream of ERK1/2, but the Raf family have been the most extensively studied. Finally MEK proteins subsequently phosphorylate members of the MAPK family. Specifically, ERK1 and ERK2 are activated by MEK1 and MEK2 respectively (Crews et al., 1992, Seger et al., 1992). MEKs are dual specificity kinases that phosphorylate ERK on a conserved tyrosine and threonine residue in the activation loop of each protein, Y185 and T183 in the case of ERK2 (Payne et al., 1991, Robbins et al., 1993). They utilise a common docking domain (CD domain) for interactions with MAPKKs, MAPKAPKS and phosphatases. This comprises an acidic cluster DXX(D/E) that follows the kinase domain and is conserved in all MAPKs (Chen et al., 2001). ERKs1/2 also have another site called ED, which is near CD and also helps to direct docking specificity.

In quiescent cells, ERK1 and 2 are cytoplasmically localised. This is mediated through their interactions with microtubules and MEK1 and 2 (Fukada et al., 1996) since MEK1 and 2 both have a nuclear export signal (NES) in their amino-terminal. Another protein that restricts nuclear entry by ERK1/2 is PEA-15, which also has a NES (Formstecher et al., 2001). Upon cell stimulation ERK, dual phosphorylation induces conformational changes which release it from protein partners and permits ERK to translocate to the nucleus. There are three pathways in operation for nuclear import of ERK: the first is passive diffusion of monomeric ERK through the nuclear pores; the second is the active

transport of dimerised ERK which requires the Ran GTPase and importin-β family of proteins; and lastly through interactions mediated by the direct contact between active ERK and the nuclear pore complex (reviewed in Torii et al., 2004). If the ERK pathway is transient then ERK will rapidly translocate out of the nucleus, but if the ERK pathway is sustained then it will accumulate. Export of ERK from the nucleus depends on dual specificity phosphatases e.g. MPK and PP2A, and reassociation with proteins containing a NES. Control of subcellular localisation of MAP kinases is also aided by scaffold proteins such as KSR, MEK partner-1 (MP1), β-arrestins, similar expression to FGF (Sef) and IQGAP, which bring components together so that they are able to interact upon stimulation or, alternatively, help to keep components apart so that they are unable to interact (Raman et al., 2007).

All MAPKs phosphorylate substrates on the consensus (threonine/serine) proline sequence. Activated ERKs1/2 have a number of substrates in various cellular compartments such as the membrane proteins, CDd120a, Syk and calnexin; nuclear transcription factors such as NF-AT, Elk-1, MEF2, C-Fos, c-Myc, SAP-1, p53, SP1, SMAD1, SMAD2, SMAD3, SMAD4 and Ets; other nuclear substrates such as SRC-1, Pax6, STAT3 and histones (which results in chromatin remodelling); MAPK interacting proteins such as RSK, MSK and MNK; and finally cytosketal proteins involved in cell adhesion and attachment such as neurofilaments, paxillin, calpain and focal adhesion kinase (Roux & Blenis, 2004, Turjanski et al., 2007). In the case of transcription factors, ERK phosphorylates them upon a transcriptional activation domain (TAD), which enhances their transcriptional activity (Ofir et al., 1990). The cell type in which ERK is activated as well as the duration and the

intensity of ERK activation will affect the substrates that it targets as explained in the following section.

1.3.1.2 Maintaining Specificity of Responses to a Receptor through control of the duration ERK MAPK signalling

The ERK MAPK pathway is utilised by a diverse range of receptors and one way in which specificity can be achieved is through control of the magnitude and duration of kinase activation (Marshall, 1995). The cell expresses a myriad of receptors that are able to stimulate this same pathway and so the cell must be able to distinguish at the nuclear level which receptor has been stimulated at the cell surface thus a receptor-defined MAPK "footprint" could be one way of achieving this.

For instance, in the PC12 system the cells express many growth factor receptors able to provoke ERK MAPK activation, thus the length of time that ERK1/2 is phosphorylated is crucial and varies depending on which receptor is stimulated. Stimulation for the same length of time with FGF or NGF triggers neurite outgrowth and halts cell division (Greene & Tischler, 1976) whereas stimulation with EGF spawns cell division (Huff et al., 1981). NGF leads to a persistent rise in Ras-GTP and results in ERK activation lasting several hours whilst EGF brings about only a short-lived rise in Ras-GTP and more transient ERK activity. Persistent ERK activation led to an accumulation of ERK in the nucleus whereas transient activation resulted in a higher quantity of cytoplasmically residing ERK (reviewed in Marshall, 1995). Recent data has suggested that a large active pool of ERK is able to translocate to the nucleus within 5 minutes of growth factor receptor ligation, but for receptors that induce only a transient ERK response, this pool is then rapidly and

permanently expelled from the nucleus through active transport (Volmat et al., 2001). Therefore at later time points it may appear that the ERK pool has remained cytoplasmic. Although prolonged MAPK pathway activation results in a nuclear accumulation of ERK, it has been found that after 3 hours of stimulation this nuclear ERK may not be in a phosphorylated form and inactive ERK may become anchored in the nucleus. This is most probably because MEK is still active upstream therefore the inactive ERK can not reassociate with the activated MEK and, because ERK does not harbour a NES, it becomes trapped (Volmat & Pouysségur, 2001). Recent studies have shown that sustained not transient ERK activation promotes expression of early gene products such as Fos, Jun, Myc and Egr-1, which are required for entry into the cell cycle. However, is now emerging that a sustained ERK signal may not always result in proliferative effects. For instance, high levels of ERK signalling can lead to cell cycle arrest by inducing expression of the CDK-inhibitors such as p21 or p27 (Dhillon et al., 2007). Furthermore, ERK can also regulate expression of proteins that promote cell cycle arrest and apoptosis in some cells. These include p53, which ERK can also serine phosphorylate at serine residue 15, and Bax, which is a proapoptotic Bcl-2 family member that enters the nucleus and promotes release of proapoptotic proteins into the intermembrane space (Reviewed in Zhang & Schnellmann, 2006). Also in HEK-293T cells it has been shown through expression of an inducible form of Raf that potentiated stimulation of ERK activates caspase-8 and increases apoptosis (Cagnol et al., 2006).

One of the main factors that can influence MAPK signalling strength and duration is the stability of the early signalling complex formed at the activated receptor (O'Rourke &

Ladbury, 2003). This will ultimately determine the length of time complexes such as Grb2-SOS persist at the cell surface and therefore the length of time that exchange of GDP for GTP occurs upon G-proteins such as Ras that go on to activate MAPKKKs. To elaborate, if a stable complex is formed Ras may be active for an extended interval whereas the Ras activity will be shorter lived if the early signalling complex is unstable and dissociates rapidly. Therefore the downstream ERK MAPK pathway will be more succinct too. Other factors influence the duration and strength of the ERK MAPK response too such as receptor density, rate of internalisation of receptors, post-translational modifications such as ubiquitination, rates of dephosphorylation, subcellular localisation of MAPKs and access to substrates (Marshall, 1995).

To summarise, the precise timing of the ERK MAPK will determine the effector response and the cell employs multiple mechanisms that work in combination to achieve this feat so that ERK is only phosphorylated for a precise duration according to which receptor has been stimulated.

1.4 Two NRTKS that utilise an overlapping subset of proteins to evoke ERK MAPK signalling with a different time course: the Interferon α receptor and T-cell receptor

In the case of both RTKs, NRTKs and other receptors such as G-protein coupled receptors, the recruitment of the correct assemblage of proteins is mandatory for eliciting the appropriate signalling cascades in response to the bound ligand at the receptor. The spatio-

temporal control of protein complex components and longevity of the complex will determine the outcome of receptor stimulation as various signalling pathways are activated for a specific duration. Additionally, as described in section 1.2.3, it is becoming apparent that additional specific signalling outcomes may be accomplished through crosstalk with other receptors expressed upon the surface of the same cell. This shows that receptor signalling involves complex networks of interactions rather than linear cascades. It is not simply a case than one receptor will activate a set of exclusive proteins that in turn activate linear cascades. Instead a given receptor will recruit proteins into a multimolecular, dynamic complex that regulates proteins downstream that may interact and co-operate with one another to induce responses and, in certain circumstances, evoke complex assembly at a totally different receptor. It was the intention to study how separate receptors, upon the same lineage of cell, can utilise a similar complex of proteins to trigger the same pathway with a different time course to bring about opposing cellular outcomes. The two receptors chosen to study, which are expressed upon the T-cell surface which are:-

(a) The T cell receptor (TCR), which causes cell growth and differentiation, and

(b) The interferon receptor (IFNR), which is involved in growth arrest, antiviral and anti-tumour effects.

These two receptors were chosen because both are fairly well characterised and it is already known that they utilise an overlapping set of the same proteins in order to initiate an ERK MAPK response with a different time course (Petricoin III et al., 1997, Lund et al., 1999, Ahmed et al., 2005). Both receptors share other similarities since both the TCR and IFNR comprise a dimeric core to which the cognate ligand binds. Furthermore, as they do not possess intrinsic catalytic kinase activity, both receptors are associated with members of

non-receptor tyrosine kinase (NRTK) family, that trans- and auto-phosphorylate specific tyrosine residues located in the cytoplasmic region of the receptor subunits. The TCR is associated with members of the Src and Syk family of NRTKs. The IFNAR is associated with members of the JAK family of NRTKs. At both receptors tyrosine phosphorylation by these kinases initiates downstream signalling events by creating docking sites through which adaptors and enzymes are recruited to the receptor. The subsequent formation of the correct assemblage of proteins at the receptors is imperative for eliciting the appropriate signalling cascades (Paccini et al., 2000). This leads to up- or down- regulation of receptor-defined sets of genes within the nucleus. The proteins recruited to each receptor and the subsequent signalling events that take place are described in detail in the following sections. The last part of the introduction describes past explorations that have shed light upon shared proteins engaged by each receptor.

1.5. Use of the T-cell receptor to study early signalling complex formation

1.5.1 The Role of T cells Within the Immune System

The immune system has two divisions: the innate immune response and the adaptive immune response. The former is a generalised, non-specific, immediate response mounted against all foreign pathogens as a first line of defence and is inherent in all living things. The latter, is a very specific response involving the recognition of precise antigen-derived peptides so that a more acute response can be mounted. Furthermore, unlike innate immunity, the adaptive immune system has “memory” i.e. after a pathogen is successfully

recognised and overcome, specialised cells are maintained called memory cells and if the same pathogen is encountered on a future occasion, the response to deal with the invader will be much faster due to the presence of these cells. The adaptive immune response is the second line of defence as it is triggered by components of the innate system through presentation of bacterial peptides. The innate immune system includes cells such as macrophages that engulf microbes, eosinophils, basophils, mast cells, natural killer cells and dendritic cells that recognise generalised motifs found in all pathogens. T cell lymphocytes are constituents of the adaptive immune response and are mandatory in the identification of invading pathogens and for mounting a specific effector response in order to overcome and destroy the pathogen. The other cell type of the adaptive immune system is B cell lymphocytes, which are able to secrete antibodies that coat microbes so that they can be recognised and destroyed. The primary receptor governing activation of the T cell is T cell antigen receptor (TCR). This senses a specific antigenically-derived peptide presented in the context of the major histocompatibility complex (MHC) by a neighbouring antigen presenting cell (APC), which can either be a B-cell or dendritic cell or macrophage. Each T cell lymphocyte is clonotypic i.e. each given cell will recognise only a specific antigenic peptide (as described further in section 1.3.2). In order to precisely modulate the activity of T lymphocytes, they express a plethora of additional receptors including co-receptors that aid antigen recognition, chemokine receptors that guide cell movement, cytokine receptors which transmit information governing positive or negative regulation of T cell growth or differentiation, and integrin receptors, which aid T-cell adherence.

1.5.2 T-Cell Receptor Development and T-cell Receptor Structure

Expression of the mature TCR complex upon a T-cell is very tightly regulated during development. The repertoire of T cells that progress to maturity, able to participate in an immune response, are carefully selected so that they express a TCR that will recognise a unique foreign antigenic peptide bound to binding cleft of an individuals own MHC molecules (Werlen et al., 2003).

T cells are derived from hematopoietic stem cells (HSCs). Pre-T-cells arrive in the thymus from the bone marrow as double negative (CD4-CD8-) precursors and complete their development in the thymus (Zlotoff et al., 2008). The first developmental stage involves progression from double negative into double positive (CD4+CD8+) T cells. This stage is driven through expression of a pre-TCR receptor, which is comprised an invariant surrogate α chain and a variant TCRβ chain. The variant chain is encoded through somatic rearrangements of V(D)J gene segments. The second stage of maturation is from the double positive (DP) CD4+CD8+ to single positive (SP) CD4+ or CD8+ T cells. The progression from the DP to SP stage is driven through expression of a mature TCR comprised of the not only the variant β chain but also a variant α chain, which is also encoded through somatic rearrangements. This variant α chain replaces the invariant α chain of the pre-TCR on the cell surface. The CD4+ subset primarily help other cells of the immune system (therefore known as T-helper cells) whereas CD8+ T cells are responsible for killing infected cells (therefore known as cytotoxic T cells). Naïve T-lymphocytes reside within the peripheral lymph nodes and remain there until presented with their cognate antigenic ligand during an infection of the body.

Each maturation stage described above is carefully orchestrated through signals received from surrounding cells. These signals must be of the correct strength and duration. Only a small percentage (approximately 3%) of T-cell precursors will develop into mature thymocytes due to the fact that they do not express a "suitable" TCR.

"Suitable" TCRs must not recognise self peptides in order to avert autoimmune disorders i.e. self-tolerant, and must also binds to a unique cognate ligand with the correct binding affinity i.e. self-restricted. The selection of T cells bearing appropriate TCRs is termed "positive selection" and cells that do not progress to maturity are either "negatively selected" or are subjected to "death by neglect", which means that the cells fail to engage a peptide-MHC complex and consequently do not receive any survival signals (Palmer, 2003).

A key determining factor that governs T cell fate is the strength of interaction between the TCR and MHC complex i.e. binding affinity (Goldrath & Bevan, 1999). If the TCR - self-peptide/MHC (pMHC) interaction is too strong then these T cells undergo negative selection and undergo will undergo apoptosis, lineage deviation or receptor editing (Starr et al., 2003). Extremely weak or absence of a TCR-pMHC results in death by neglect due to insufficient survival signals. Cells which are positively selected are those bearing a TCR that engages the pMHC somewhere in the middle of these two extremes. Not only does the binding affinity affect T cell survival but also the number or avidity of pMHC-TCR interactions. A low number of high affinity TCR-pMHC interactions results in negative selective whereas a high number of low affinity TCR-pMHC interactions results in positive selection (reviewed in Palmer & Naeher, 2009).

At each point of selection the pre-TCR and mature TCR, the quantitative signalling attributes of the TCR help to govern survival and a primary pathway is activated downstream of the TCR is the ERK MAPK cascade. The kinetics of this pathway have been found to hold a profound significance in the determination of T cell fate during development (Werlen et al., 2000). Studies have shown that during negative selection ERK is activated rapidly and transiently due to a strong TCR interaction with its ligand whereas positive selection, which requires a weaker TCR-ligand interaction, results in ERK being phosphorylated much more slowly (Werlen et al., 2000). In addition, the intensity and duration of ERK activation downstream of the TCR affects whether double positive T-cells differentiate into the CD4+ or CD8+ single positive lineage. Weak ERK MAPK results in cells developing into CD4+ T cells whereas stronger ERK MAPK signalling causes cells to enter the CD8+ lineage (Singer et al., 2002). Other MAPK signalling pathways bare a particular significance in negative selection also. The stress-induced MAPKs, p38 and JNK MAPKs, are triggered in negatively selected cells, which contribute to eventual cell death.

In T cells Ras can be activated by two different GEFs; Sos or RasGRP. RasGRP is DAG-dependent exchange factor and therefore relies upon upstream PLCγ activity for DAG generation. It has been found using RasGRP1-/- mice and various knockins of these mice that the CD4+ lineage relies upon ERK phosphorylation generated through the exchange factor RasGRP rather than Ras. Since this exchange factor is activated more slowly than Ras since it relies upon upstream PLCγ activity, this is thought to account for the observation that CD4+ lineage relies upon weaker, protracted ERK activation (Priatel et al., 2006). Due to the fact that T-cell survival relies heavily upon (pre)TCR-generated signals, TCR-associated molecules such as Lck, Fyn, LAT, Zap70, Vav and Slp76 are all

imperative and mice lacking these components display partial or total blocks in thympoiesis. These molecules are required for the generation of downstream ERK signals so affect positive/negative selection as well as commitment to the CD4 or CD8 single positive cell type. These proteins and their roles in mature T-cell activation are discussed in more depth in future sections.

The mature TCR complex upon positively selected cells is a large structure that comprises eight chains in total. The central core consists of a disulphide-linked heterodimer composed of the α and β chains in the majority cases. A small subset of T cells express a γδ heterodimer and are required for different immune functions. Each αβ heterodimer dictates antigenic peptide recognition in the context of the MHC complex. The chains have a short intracellular region, a single transmembrane spanning domain and an extracellular immunoglobulin domain that encompasses both a constant region that is identical between all T cells and a variable region, which confers the chains with their antigenic specificity.

The αβ chains are non-covalently associated with six other chains known as the CD3 complex, which are invariant and constitute the intracellular signalling machinery since the α and β chain are devoid of this capacity due to their very short intracellular region. Each chain is tethered to the cell membrane via a single transmembrane domain. The six chains of the CD3 complex are arranged into three dimers: γε, δε and ζζ (Alarćon et al., 2003). The cell surface expression of the αβ chains relies upon these associated CD3 complex signalling subunits (Brenner et al., 1985, Samelson et al., 1985). The extracellular domains of the CD3δε and CD3γε heterodimers consist of side-by-side paired Ig folds whereas the ζζ homodimer only has a nine amino acid extracellular region (Kuhns et al., 2006) - See figure 1.3.

The CD3 complex contains ten specialised signalling motifs know as immunoreceptor tyrosine-based activation motifs (ITAMs). Six of these are spread equally between the ζζ chain homodimer (three on each chain) and each of the other chains of the CD3 complex containing one ITAM each (Pitcher & van Oers, 2003). ITAM motifs are not unique to the TCR but can also be found within other immunoreceptors such as the B-cell receptor and Fc receptors that recognise the Fc portion of antibodies during antibody dependent cytotoxicity. An ITAM is defined by two tyrosine residues contained in the sequence: YxxI/Lx(6-12)YxxI/L (Underhill & Goodridge, 2007). Upon phosphorylation of key tyrosines encompassed within the ITAM motifs, the phospho-tyrosines act as high affinity docking sites for proteins involved in the early signalling complex, so are therefore accountable for the initiation of intracellular signalling and mediating downstream responses.

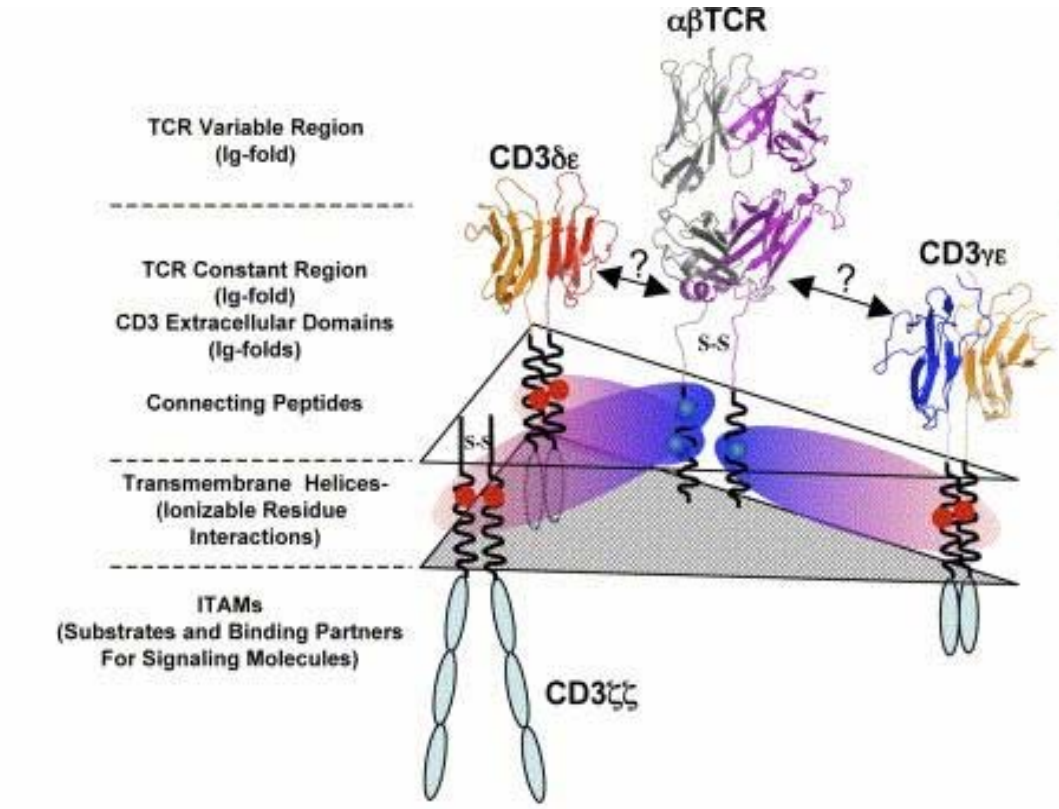


Figure 1.3 TCR Receptor Structure ***(Taken from Kuhns et al., 2006)***

The TCR is comprised of a dimeric αβ core that is associated with six CD3 complex chains arranged into three dimers: ζζ, γε and δε.

1.5.3 Initiation of T-cell Signalling and Proteins Involved in the Early Signalling Complex at the Receptor

The proximal events following ligation of the T-cell receptor (TCR), expressed on the surface of T-cells, have been extensively studied over the last decade or so. The adaptors and enzymes involved at the stimulated TCR are extremely well-characterised and form a large, dynamic ensemble, binding to multiple partners simultaneously.

The TCR lacks intrinsic tyrosine kinase activity and instead relies on the deployment of NRTKs, which are able to phosphorylate specific tyrosine residues of the ITAM motifs. The TCR complex, the CD4 co-receptors, transmembrane adaptor proteins and the NRTKs are located within lipid rafts, which are submicroscopic regions of the lipid bilayer that are enriched in sphingolipids and cholesterol. These rafts compartmentalize the molecules required for T cell receptor signalling. Two signals are required for full activation of naïve T-cells; the first is achieved through recognition of the MHC bound peptide by the TCR, and the second is provided through the interaction of the CD28 co-receptor with members of the B7 family of receptors upon the APC, more specifically B7-1 and B7-2. Both signals are needed in a physiological environment to achieve full T cell activation. However, in T cell lymphoma cell lines that are already dividing, signalling can be observed through activation of the TCR only with an antibody that binds it.

Upon TCR engagement with a cognate peptide in the context of the MHC complex, Src family kinases, such as Lck and Fyn, redistribute and phosphorylate two critically spaced tyrosine residues 9-11 amino acids apart, located in the ITAMs of the ζ chains. The TCR co-receptor, CD45, is responsible for activating Lck. CD45 is a transmembrane phosphatase that dephosphorylates the negative regulatory site of Lck and also

dephosphorylates Cbp/Pag thus releasing the negative-regulator, Csk, which is no longer within close proximity to Lck hence enabling full Lck activation. In addition, a portion of Lck is also constitutively associated with the CD4 or CD8 coreceptor, which is able to cause physical increases in Lck concentration around the TCR through this association thus aiding the rapid phosphorylation of ITAMs (reviewed in Hermiston et al., 2002).

The phosphotyrosines of each ITAM serve as high affinity docking sites for the tandem SH2 domains of members of the Syk family of protein tyrosine kinases, most importantly the key effector Z-associated protein of 70kDa, Zap70 (Chan et al., 1992) and also, in T-cells that lack CD4 or CD8 co-receptors e.g. in the gut epithelium, the family member, Syk (Chu et al., 1996). Zap70 is activated by trans- phosphorylation by Lck on its activation loop at Tyr-493 and this activation by Lck causes Zap70 to auto-phosphorylate further upon residues Tyr-292, Tyr-315 and Tyr-319 (Chan et al., 1995 & Wange et al., 1995). The phosphotyrosines on Zap70 then become key docking sites for SH2-containing or PTB-containing proteins and importantly, activated Zap70 is able to phosphorylate a restricted subset of proteins e.g. Vav1 (Wu et al., 1997), Shc (Pacini et al 1998), LAT (Zhang et al., 1998) and Slp76 (Wardenburg et al., 1996). LAT and Slp76 are multi-domain proteins that act as key adaptors/molecular scaffolds in T-cell signalling once phosphorylated and are able to arbitrate the formation of multi-molecular signalling complexes through binding of an array of proteins. They are unable to bind one another directly, but instead utilise GADS protein which forms a crosslink between the two. LAT is an essential membrane-spanning adaptor protein with a short extracellular region and a long tyrosine-rich cytoplasmic tail (Zhang et al., 1998) and contains binding sites for Grb2, GADS and PLCγ, which is crucial for phospholipid metabolism (Zhang et al., 2000). Slp76 is an intracellular adaptor protein

capable of binding GADS, ADAP (Raab et al., 1999) and also Vav1 (Wu et al., 1996) and Nck (Bubeck Waldenburg et al., 1998) proteins which are vital for cytoskeletal changes as well as a number of other downstream responses. It can also become phosphorylated by and bind to the Tec kinase, Itk (Bunnell et al., 2000 & Schneider et al., 2000), which is another cytoskeletal intermediary.

Further adaptors, scaffolds and enzymes are also recruited to phosphotyrosines both on other proteins and the TCR itself such as the p85 subunit of PI3K and Grb2. Together, all of the mentioned proteins form a complicated assemblage around the T-cell receptor. See Fig. 1.4 for a diagram for of the main early signalling complex components as well as the signalling pathways activated by these proteins. Many experiments have been performed that substantiate the idea that a multi-molecular complex forms at the TCR. Paccini et al., 2000, showed that the early signalling complex linked to the TCR is dynamic, with each protein forming many interactions. They showed, through a series of immunoprecipitations for eleven proteins conducted at different time points that constituents assemble and disassemble at discrete intervals. Each of the proteins highlighted is differentially phosphorylated and persists in the complex for a regulated duration. For example, Zap70 maximally associates with the ζ chain between 15 seconds and 2 minutes. Furthermore, there are many recent papers that utilise new advancements in confocal imaging to trace the localisation of fluorescently tagged proteins and employ techniques such as fluorescence resonance energy transfer (FRET) and total internal reflection microscopy florescence (TIRF) that validate the interactions between T cell receptor proteins (Bunnell et al., 2002., Yokosuka et al, 2005, Bunnell et al., 2006, Barr et al., 2006) For example, Zap70 forms part of microclusters around the TCR ζ chain and persists there for between 20 and 30

minutes. Slp76 is also a component of these microclusters but only persists at the cell surface for a limited period and instead moves to peri-nuclear compartments along microtubules (Bunnell et al., 2002, Barr et al., 2006).

The formation of a carefully orchestrated complex around the TCR results in a number of crucial signalling events, which are explored in more detail in the following section (1.5.4). Adaptors, kinases and phosphates enter and leave the TCR complex in order to tightly regulate downstream events. The molecules involved in TCR signalling are described one by one in section 1.5.6. The events required to down regulate signalling are described in section 1.5.5.

1.5.4 Signalling Pathways Activated During T-cell Signalling

There are three main effector pathways brought about by ligation of the TCR: MAPK signalling, phospholipid metabolism and calcium flux. This leads to induction of various transcription factors and immediate early genes and late genes that bring about the correct response by the cell. This is also coupled with many cytoskeletal changes and reorganisations. Within 30 minutes, transcription factors such as NFAT, NFκB, *c-jun*, *c-fos* and *c-myc* are induced and the protein expression of these immediate early genes in turn activates one of the most crucial genes transcribed upon TCR provocation, which is the IL-2 gene. Once transcribed, the IL-2 cytokine is then secreted and acts in an autocrine and paracrine manner to augment the G1-S phase transition of the cell cycle in TCR-activated T cells through the IL-2-IL-2R interaction, thus allowing clonal expansion and cellular differentiation (Cantrell & Smith, 1984, Smith, 1988) into effector T cells.

1.5.4.1 MAPK

This pathway is described in more detail in section 1.3. At the TCR, the primary MAPK cascade activated and responsible for cellular proliferation is the ERK MAPK cascade. In addition, the p38 and JNK MAPK pathways are also induced. ERK MAPK signalling is also important in the positive selection of thymocytes during development whereas the JNK MAPK pathway plays a role in negative selection (Werlen et al., 2000).

This is especially important physiologically in promoting clonal expansion of primed T-cells during an adaptive immune response. TCR ligation leads to a rapid accumulation of the active GTP bound Ras at the cell membrane. GDP/GTP exchange on Ras can be inititiated through two routes; Grb2-SOS mediated Ras activation or through activation of RasGRP, a DAG-dependent exchange factor for Ras (Dower et al., 2000, Roose & Weiss, 2000). In addition, Grb2 simultaneously binds Zap70 (Nel et al., 1995), Shc (Ravichandran, 1995), LAT (Zhang et al., 1998) and Vav (Ye & Baltimore, 1994, Nishida et al., 2001, Ogura et al., 2002). This signalling complex formation stabilises interactions needed or Ras activation, which triggers the Raf-1/MEK/ERK signalling cascade. This leads to activation of the transcription factors such as activator protein 1 transcription factor (AP1), c-Fos and also the transcription factor c-myc, which are involved in transcriptional regulation at the IL-2 promoter (Cantrell & Smith, 1984).

The JNK1 and JNK2 isoforms are present in low quantities in quiescent T-cells, but upon TCR stimulation the expression of these MAPKs increases significantly after 30 minutes (Weiss et al., 2000). The co-receptor CD28 is also required for JNK activation and JNK in turn is required to activate transcription factors at the IL-2 and IFN-γ promoters (Su et al.,

1994). The p38 cascade also contributes to IFNγ gene induction, but this only occurs in the T_H1 subset of CD4+ T cells, which are responsible for producing large amounts of IFNγ.

1.5.4.2 Phospholipid Metabolism

The TCR is coupled to PLCγ and PI3K, which are enzymes responsible for catalysing the breakdown of different phospholipid substrates located within the membrane (Acuto & Cantrell, 2000). This is for generation of important second messenger molecules, required for amplifying the TCR signal.

PLCγ requires three PTKs for phosphorylation at the T cell antigen receptor; Lck, Zap70 and Itk (Liao et al., 1993, Law et al., 1996). Also it binds to both LAT, through its SH2 domain (Finco et al., 1998), and Slp76 (Yablonski et al, 1998), which are interactions necessary for its stabilisation within the early signalling complex. PLCγ is responsible for the hydrolysis of phosphatidylinositol (4,5) bisphosphate (PIP2) to inositol triphosphate (IP3) and diacylglycerol (DAG). DAG also regulates Protein kinase C (PKC) isoforms, which are serine/threoinine kinases. In T-cells, the PKCθ isoform is recruited to the APC-TCR interface and helps propagate signals that regulate NFκB activity (Cronin & Penninger, 2007). DAG is also capable of activating the Ras guanisine exchange factor, RasGRP, which in turn promotes the exchange of GDP bound to the membrane-bound G-protein, Ras, for GTP. Therefore DAG in directly promotes downstream MAPK activity (Dower et al., 2000, Roose & Weiss, 2000). IP3 is capable of binding to receptors upon the endoplasmic reticulum that leads to the release of calcium ions (see next section: 1.4.4.3). PI3-K is heterodimer of a p85 regulatory subunit and a p110 catalytic subunit. PI3-K phosphorylates the D-3 position of the inositol head group of phophatidylinositol (4,5)

diphosphate (PIP2) to produce phophatidylinositol (3,4,5) triphosphate (PIP3). Generation of PIP3 leads to recruitment of a number of important cytoskeletal proteins such as Vav (Han et al., 1998, Zugaza et al., 2002) and Itk (Yang et al., 2001), through the interaction of PIP3 with PH-domain-containing proteins. PIP3 is also important for downstream activation of Akt/PKB and in the activation of PDK (phosphoinositide-dependent kinase).

1.5.4.3 Calcium Flux/ NFAT

IP3 binds to receptors located on the ER (endoplasmic reticulum) and this causes the calcium ions stored within this organelle to be released into the cytoplasm of the T-cell. Rises in levels of intracellular calcium also causes store-operated calcium release-activated calcium (CRAC) channels to open with the plasma membrane allowing more calcium ions into the cell. This is known as the calcium flux as intracellular calcium levels suddenly rise rapidly following TCR ligation and the elevation in calcium levels may persist for a period of minutues to hours. Increases in intracellular calcium ions leads to activation of a calcium/calmodulin-dependent phosphatase called calcineurin. Activated calcineurin dephosphorylates multiple serine residues located within the regulatory region of the NFAT transcription factor (Loh et al., 1996). The NFAT family of transcription factors has five members (NFATs1-5). This induces a conformational change in NFAT that exposes its nuclear localisation signal and allows NFAT to be translocated to the nucleus (Okamura et al., 2000). NFAT is translocated in concert with calcineurin through nuclear pores. Within the nucleus, NFAT interacts with cofactors to form transcription factor complexes, e.g. members of the Fos, Jun and GATA familes (Katzav, 2004). The interactions in the T-cell drive the transcription of the IL-2 gene (Shaw et al., 1998), allowing T-cell proliferation

and clonal expansion during an adaptive immune response. NFATs have also been associated with the inducible of expression of a host of other genes such as IL-3 (Cockerill et al., 1993), IL-4 (Chuvpilo et al., 1993), IL-5 (Stranick et al. 1997) , granulocyte-macrophage colony-stimulating factor (Cockerill et al., 1993), IFNγ (Campbell et al., 1996), FasL and tumour-necrosis factor-α (Falvo et al., 2000).

The association of NFAT with co-factors is under the strict regulation of a number of kinases such as JNK1, JNK3, ERK, glycogen synthase kinase-3 and protein kinase A (PKA) that rephosphorylate NFAT, thus re-exposing its NES causing relocalisation back to the cytosol (Quitana et al., 2005).

1.5.4.4 NFκB

A key step in the activation of the transcription factor, NFκB, is the activation of protein kinase C θ (PKCθ). This PKCθ activation is controlled by events within the early signalling complex as PKCθ can be activated by DAG, a product of PLCγ activity, as well as by pathways initiated through activation of Rac, which is activated upstream by the GEF activity of Vav1 (Villalba et al., 2000). PKCθ promotes formation of the IKK complex, which is comprised of an α,β and the regulatory subunit, γ (also known as NEMO). This complex then phosphorylates IκB, which is a negative regulator of NFκB transcription factor. Phosphorylation of IκB releases it from its bound STATe exposing a nuclear localisation signal upon NFκB, thus allowing this transcription factor to translocate into the nucleus, where it can regulate target genes (Huang & Wange, 2004). Within the nucleus NFκB is able to form a transcriptional complex with p65, c-rel and relB (Weil & Israel,

2004). Another important target for the NFκB pathway is the Bcl-x_L promoter, which when expressed plays a critical role in cell survival (Khoshnan et al., 1999).

1.5.4.5 Cytoskeletal reorganisation

The contact surface formed between the antigen presenting cell and T-cell is a complex synapse. This can last for hours and consists of many integrin receptors and cytoskeletal components. In order for the synapse to form cells polarise, upregulate a host of molecules and undergo cytoskeletal reorganisations. Cytoskeletal changes are also required in order for a T-cell to migrate to where it is needed within the body. This requires lamellipodia, which are actin-rich protrusions and actin polymerisation (Hornstein et al., 2004). The actin polymerisation of filamentous actin (F-actin) is mediated by a complex of WASP/WAVE and the Arp2/3 proteins (Miletic et al., 2003).

Vav and Slp76 are particularly important for cytoskeletal rearrangements. Vav activates Rac/Rho proteins through its catalytic GEF activity which then mediate downstream actin polymerisation, the formation of lamellopodia and membrane ruffling (Villalba et al., 2001). Wiscott-Aldrich Syndrome protein (WASP) is a downstream effector of Rho GTPases. The VCA domain of WASP is responsible for interacting with the Arp2/3 complex and actin nucleation. The domain is regulated by Cdc42-GTP binding to the GTPase binding domain and WASP activity is also regulated through binding to WASP Interacting protein, WIP (Miletic et al., 2003). Slp76 can recruit Nck, which in turn recruits PAK1, which is then able to bind WASP, initiating formation of complexes than control actin polymerisation. Slp76 also recruits an adaptor called adhesion and degranulation-

promoting adaptor protein (ADAP), which associates with another protein called SKAP-55 and, together, this complex plays a role in upregulation of integrin receptors in order to stabilise the TCR-APC synapse (Musci, 1997b, Zeng et al., 2003). In addition, Itk helps to arbitrate molecules involved downstream cytoskeletal changes by nucleating the complex formed between LAT, PLCγ, Vav and Slp76 (Finkelstein & Swartzberg, 2004). Itk deficiency affects β1-mediated adhesion of Jurkat cells also (Woods et al., 2001).

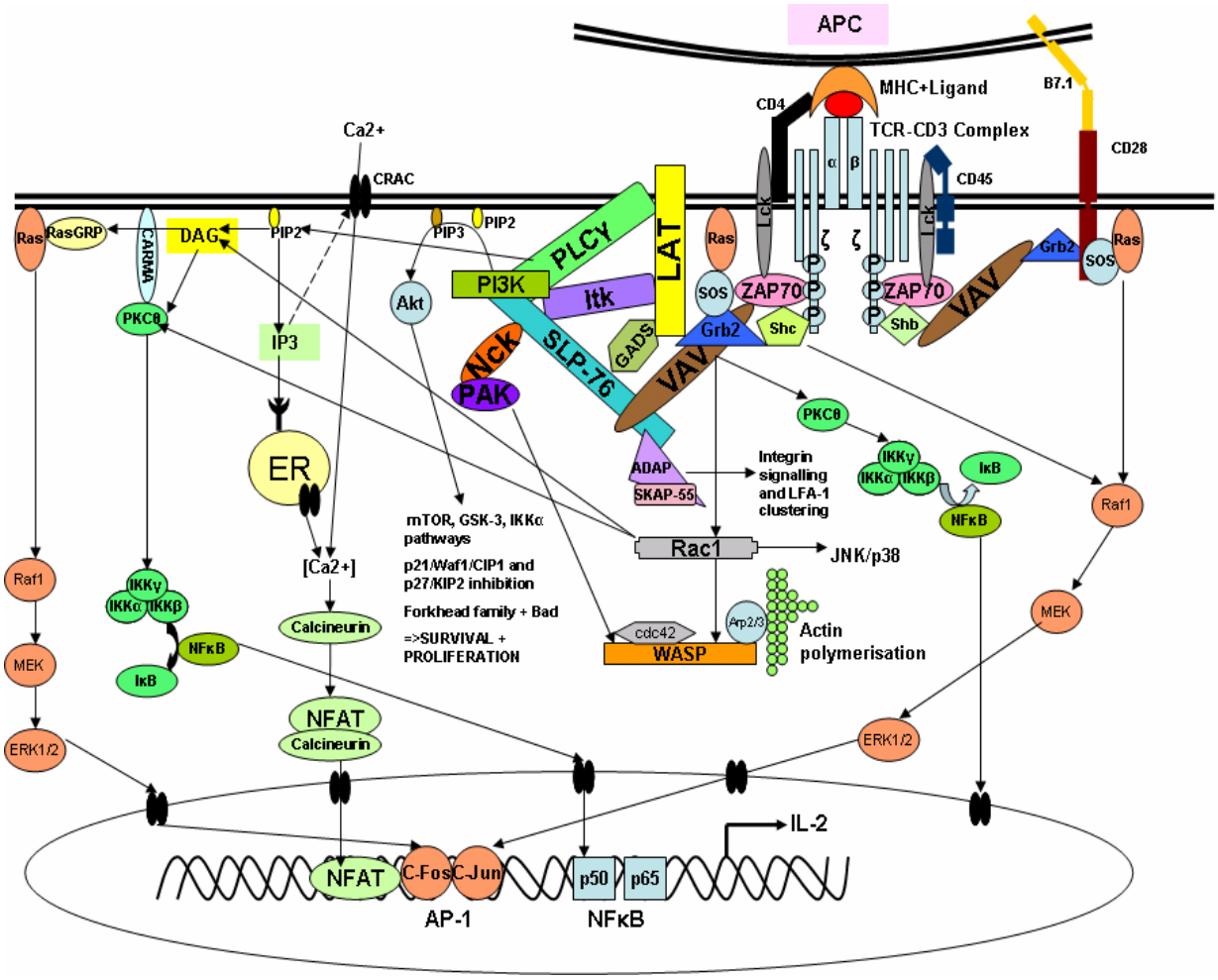


Figure 1.4 TCR Signalling

An early signalling complex is recruited to the TCR upon its recognition of an MHC-bound antigenic peptide on the surface of an antigen presenting cell. This leads to a number of key signalling cascades, which result in cytoskeletal changes and upregulation of genes that promote cellular growth and differentiation

1.5.5 Downregulation of Signalling at the TCR

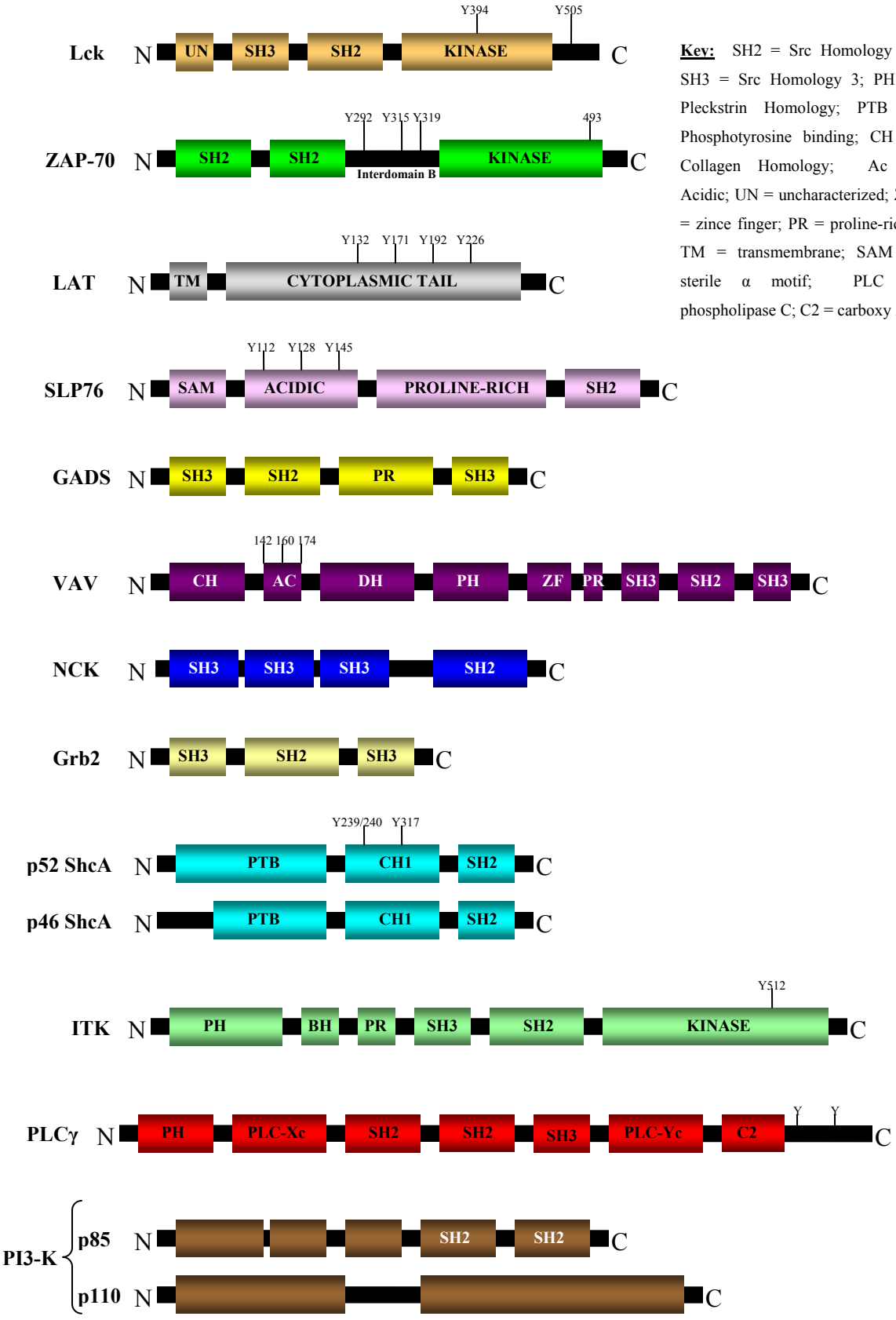
There are two families of protein phosphatases that counterbalance the phosphorylation events induced by TCR activation; the serine/threonine phosphatases e.g. PP1 and PP2, and protein tyrosine phosphatases (characterised by a highly conserved catalytic phosphotyrosine phosphatase domain). Additionally some phosphatases may be dual specificity phosphatises that possess both phospho-serine/-threonine and phosphotyrosine catalytic activity e.g. those that down regulate MAPK signalling. T-cells express at least 40 different PTPs, but the regulation of many of them is still poorly understood (Mustelin et al., 2004). One of the most important PTPs in initiating TCR signalling is CD45 as it dephosphorylates Lck and so has a positive influence over signalling, but there are many other PTPs that negatively regulate TCR signalling.

There are a number of transmembrane adaptors that negatively regulate T cell signalling such as PAG and SIT. PAG, which is also known as Cbp, is found in lipid rafts and is palmitoylated, with a similar structure to LAT (Wilkinson et al., 2004). It binds Csk in resting T cells, which is a negative regulator of Src kinases such as Lck. It constitutively phosphorylates Lck so that it adopts an inactive conformation, but in active T-cells PAG is transiently dephosphorylated by CD45 so that it releases Csk, thereby permitting TCR signalling to ensue (Brdicka et al. 2000). SIT is another negative regulator of TCR signalling, which mediates Grb2 binding and Src family kinase binding. It can be found at the membrane and has a short extracellular region of 18 amino acids. It also has five phosphorylation sites, including an ITIM motif (Immunoreceptor tyrosine-based inhibition motif) and binds to the phosphatase, SHP-2. SHP-1 and SHP-2 are both important phosphatases in the regulation of TCR signalling. SHP-1 dephosphorylates Zap70 and Syk

thus quenching their catalytic ability and also dephosphorylates Vav and Slp76. There are numerous other phosphatases that regulate components of the early signalling complex formed at the TCR such as the phosphatase PTP-PEST that dephosphorylates Shc, paxillin, Cas, Pyk2 and Fak as well as the transmembrane PTP CD148 that dephosphorylates PLCγ and LAT (reviewed in Koretzky & Myung, 2001).

Aside from proteins that dephosphorylate TCR signalling machinery, there are also proteins that aid in targeting substrates for degradation (reviewed in Jang & Gu). One way of targeting proteins for degradation is through the addition of ubiquitin entities i.e. ubiquitination. A family of proteins that ubiquitate TCR associated proteins are the Cbl family, which has three members: c-Cbl, Cbl-b and Cbl-3. c-Cbl can downregulate Zap70 whereas Cbl-b can ubiquinate the p85 subunit of PI3K (Fang et al., 2001) and also Vav (Bustelo et al., 1997). Cbl-b and c-Cbl can also interact with CIN85 and the clathrin-coat component endophilin in other cell types to mediate internalisation of receptors, but it is still uncertain whether the Cbl family interactions determine internalisation of the TCR (Rangachari & Penninger, 2004). Recently it has been shown that NRTKs serve a dual role in TCR signalling and not only upregulate signalling, but also help to modulate down-regulation of the ζ chain of the TCR. In cells lacking Zap70, the TCRζ chain persist for longer periods upon the cell surface whereas Zap70 positive cells undergo TCRζ chain down-regulation. This may be because Zap70 helps to stabilise other proteins that down-regulate the chain or that it activates proteins needed to down-regulate this chain through phosphorylation, but further work is required in order to characterise the role of Zap70 (Dumont et al., 2002). To summarise this section, degradation and dephosphorylation terminate TCR-induced signalling.

Figure 1.5 Modular organisation proteins utlised in T-cell receptor signalling



Key: SH2 = Src Homology 2; SH3 = Src Homology 3; PH = Pleckstrin Homology; PTB = Phosphotyrosine binding; CH = Collagen Homology; Ac = Acidic; UN = uncharacterized; ZF = zince finger; PR = proline-rich; TM = transmembrane; SAM = sterile α motif; PLC = phospholipase C; C2 = carboxy 2

1.5.6 Summary of Modular Organisation of Key Enzymes and Adaptors Involved in early TCR Signalling and description of their involvement within the early signalling complex

In this section, some of the key signalling proteins involved in TCR signalling are described. Many of the proteins contain similar domains such as SH2, SH3, PH, PTB and kinase domains. A diagram of the modular organisation of the protein discussed in each section is in Figure 1.5. Since in this thesis Slp76 and Vav are examined in more detail, their role at the TCR is discussed more comprehensively than the description of the other signalling proteins.

1.5.6.1 Lck

Lck is 56kDa NRTK belonging to the Src family of NRTKs. Another Src family NRTK, Fyn, which has a similar domain organisation, is also expressed in T-cells but it is thought that Lck plays the prominent role in initiating phosphophorylation of ITAMs upon the TCRζ chains. Fyn only displays high affinity to one ITAM since the Lck deficient Jurkat cell line, J.Cam1, results in minimal but detectable tyrosine phosphorylation (Mustelin & Tasken, 2003). Lck is anchored to the cell membrane. Both myristoylation and palmitoylation at the N-terminal thus allow this attachment. A unique region (UN) enables Lck to interact with the co-receptors CD4 and CD8 which are both required for full T cell activation. Lck is regulated through its C-terminal domain. In resting T-cells Lck exists in a phosphorylated STATe at tyrosine 505 at the C-terminus. This causes the C-terminal phosphotyrosine to interact with its own SH2 domain thus rendering Lck in a closed conformation STATe whereby the kinase domain can not access its substrates (Zamoyska

et al., 2003). Upon T-cell antigen recognition, Lck, which is constitutively associated with the CD4 or CD8 co-receptor, is brought within close proximity of the TCR. The CD45 TCR-associated co-receptor can then dephosphorylate Y505 leading to an open conformation and exposure of the kinase domain. It is the thought that trans-phosphorylation then occurs between Lck molecules recruited to the activated TCR creating a phosphorylated tyrosine at position 394 (Palacios & Weiss, 2004).

1.5.6.2 Zap70

Zap70 is a Syk family NRTK of 70kDa. The related NRTK, Syk, is also expressed within T-cells but it is Zap70 that plays the major role at the TCR. Absence of Zap70, as shown in the P116 Jurkat cell line deficient in Zap70 expression, results in severely reduced protein tyrosine kinase phosphorylation and a failure to induce calcium flux, ERK1/2 phosphorylation and IL-2 promoter driven IL-2 transcription (Williams et al., 1998, Shan et al., 2001). Zap70 phosphorylates a number of downstream targets such as Vav, Slp76 and LAT (Michel et al., 1998) and also helps to recruit Grb2 and SOS to the membrane. In fact in unstimulated cells, it has been shown that Zap70 already exists in some preformed complexes with Vav, Grb2 and SOS. The amount of these complexes is increased upon TCR stimulation (Salojin et al., 1999). Zap70 can also bind to the SH2 domains of an array of proteins via phosphorylated Y315, which is critical for receptor-mediated transduction and this YESP site is crucial for binding to Vav, Slp76 and Shc (Wu et al., 1997, Walk et al., 1998). Tyrosine 315 has also been shown to bind to CrkII, which is involved in termination of signalling from the TCR (Gelkop et al., 2005).

1.5.6.3 LAT

LAT was first cloned in 1998 by Zhang et al. It is a protein of 233 amino acids that has a very short extracellular tail, a transmembrane region and a long cytoplasmic tail that contains nine tyrosine residues. The best characterised residues are shown on the diagram below. LAT lacks any structural domains within the cytoplasmic tail. This tail contains many negative residues, which results in it running at a higher apparent molecular weight on a SDS-PAGE gel. The protein is infact 25kDa but it runs at 36-38kDa (Zhang & Samelson, 2000). LAT is palmitoylated upon two conserved cysteines at the N-terminus, allowing its association with the cell membrane. LAT contains nine tyrosine residues upon the cytoplasmic tail, the four of which that have been best characterised through mutational analyses are indicated on the diagram below (Houtman et al., 2005).

LAT contains five Grb2 binding motifs, which couples LAT to MAPK activation. LAT also interacts with the SH2 domains of both PLCγ and the p85 subunit of PI3-K. Y171, Y191 and Y226 are essential for binding to Grb2, p85 of PI3-K and GADS and Y132 is required for PLCγ binding (Zhang & Samelson, 2000). In addition LAT also interacts with other signalling proteins such as Grap, an adaptor homologous to Grb2, c-Cbl, 3BP2, Itk and Shb, another adaptor thought to link Zap70 and LAT. There is also doubt over whether it directly or indirectly binds Vav. Studies in the LAT-deficient cell line J.Cam2 have exposed LAT as being mandatory for TCR-mediated calcium flux, optimal tyrosine phosphorylation of PLCγ, Vav and Slp76 as well as being essential for ERK1/2 activation and CD69 upregulation (Zhang et al., 1999). Studies that have utilised fluorescently tagged LAT constructs have demonstrated that LAT is recruited to TCR microclusters within 15 seconds of TCR stimulation. LAT persists at the cell surface for 2-3 minutes before

appearing in small vesicular intracellular structures (Bunnell et al., 2002). Two pools of LAT exist within the cell. The first is membrane localised and the second is intracellular localised and is cycled between endosomes. When the first pool of membrane localised LAT disperses from the cell surface during TCR signalling after 2-3 minutes, it is replaced by the intracellular pool, which then persists upon the membrane (Bonello et al, 2004).

1.5.6.4 Slp76

Slp76 is a cytosolic, 76kDa, 533 amino acid adaptor protein that was first cloned in 1995 by Jackman et al. whilst screening for TCR-induced protein tyrosine kinase activity. They found that it associated with GST-tagged Grb2 in vitro and that the SH2 domain of Slp76 co-immunoprecipitated with tyrosine phosphorylated PLCγ in Jurkat cell lysates that has been stimulated through the TCR. Slp76 is exclusively hematopoietically expressed and can be found in platelets, neutrophils, mast cells, macrophages, natural killer cells and developing B-cells (Koretzky et al., 2006). Slp76 is a member of a family that includes the 65kDa B-cell linker protein, Slp-65 (also known as BLNK, BASH or BCA) and the cytokine-dependent hematopoietic linker protein, Clnk/MIST. Only Slp76 is expressed in T-cells however, meaning that the other members of the family are not involved in T-cell signalling.

Slp76 has three domains. The sterile α motif (SAM) is the most N-terminal region followed by an acidic region at the N terminus containing three key tyrosine residues at positions 112, 128 and 145 (YESP, YESP and YEPP) which are phosphorylated in a Zap70 manner (Fang et al., 1996, Raab et al., 1997). The phosphotyrosines at positions 112 and 128 mediate binding to Vav (Raab et al., 1997). They also interact with the Nck adaptor protein

(Wunderlich et al., 1999) and the p85 subunit of PI3K (Shim et al., 2004). Phosphotyrosine 145 mediates the interactions with Itk, a Tec family kinase protein that mediates cytoskeletal reorganisation (Su et al., 1999). A core RxxK motif located within the proline-rich region of Slp76 is responsible for the interaction with the SH3 domain of GADs. This interaction occurs with very high affinity and specificity, as shown through crystal structural studies, and is required for downstream NFAT activation (Lui et al. 1999, Dimasi, 2007, Seet et al., 2007). The proline-rich region also binds to PLCγ (Yablonski et al., 2001). A small region distal to the acidic region at the boundary of the proline-rich region has been reported to bind Lck (Sanzenbacher, 1999). The SH2 domain binds to phosphorylated ADAP (SKAP-130/Fyb) and HPK-1, which is a serine-threonine kinase (Musci, 1997b, da Silva, 1997, Sauer et al., 2001).

Initial experiments that shed light upon the role of Slp76 in T-cells were performed through overexpressing Slp76 in Jurkat cells. These experiments revealed increased ERK phosphorylation and augmented NFAT activation and AP-1 binding upon TCR stimulation (Musci et al., 1997a). Since then analysis of Slp76 knockout mice has further illuminated the requirement of Slp76 for full TCR signalling and correct T-cell development. Its absence leads to profound defects in both of these facets. Slp76 negative mice exhibit a block in the transition from double negative to double positive T-cells during thymic development and hence display a complete absence of peripheral single positive ($CD4^+$ or $CD8^+$) mature T-cells. This block could not be overcome by treatment with anti-CD3 and can therefore be explained by the fact that Slp76 is requisite for pre-TCR signalling and defective signalling halts further T-cell development (Clements et al., 1998, Pivniouk et al., 1998).

Since Slp76 knockout mice bore no mature peripheral T cells, studies of Slp76 involvement in TCR signalling were carried out in a Slp76-deficient Jurkat cell line, J14. This revealed that the absence of this protein hampers downstream IL-2 transcription when the TCR is ligated, compared with wild-type Jurkat cells. ERK activation was also significantly curtailed, due to defective Ras activation. TCR-induced phosphorylation of PLCγ was also defective in J14 cells. In addition the calcium flux normally seen following TCR stimulation was reduced (Yablonski et al., 1998). Furthermore, it has since been shown that downstream PKCθ and NFκB activation is defective in J14 cells (Dienz et al., 2002). Nearly all of these phenotypes in Slp76 deficient cells are shared by Vav deficient cells as both proteins bind one another and co-operate to stabilise the binding of further proteins in order to control downstream responses. Through their direct binding interactions, Vav and Slp76 mediate signalling not only through the TCR but also the CD28 co-receptor and co-operate to induce IL-2 transcription and IL-4 transcription (Koretzky & Fang, 1999, Raab et al., 2001, Rudd & Raab, 2003). In combination with Vav, Slp76 also orchestrates cytoskeletal changes upon TCR perturbation. It associates with Nck, which also binds to PAK1 and WASP, which then orchestrates actin polymerisation and actin capping. The association of the ADAP adaptor protein with the SH2 domain of Slp76 is thought to be required for 'inside-out' upregulation of the LFA-1 and VLA-4 integrins (Koretzky, 2004).

In resting T-cells Slp76 is constitutively associated with GADs in a cytosolic complex. Upon TCR engagement, this complex is recruited to LAT-containing lipid rafts, where the SH2 domain of GADS binds to phosphorylated LAT thus linking Slp76 and LAT (Singer et al., 2004). Phosphorylated Slp76 then forms a complex with Itk and PLCγ, which also bind to LAT. This complex formation is mandatory for activation of PLCγ and therefore its

role in phospholipid metabolism (Qi & August, 2007). Slp76 recruitment to the cell membrane following TCR cross-linking has also been tracked through observations of fluorescently tagged Slp76. Slp76 is initially recruited to TCRβ rich clusters that also contain GADs, LAT, Grb2, Cbl and ZAP70. Also, Slp76-rich clusters have been shown to translocate radially along microtubules 1-3 minutes after TCR stimulation to peri-nuclear structures that do not stain for endosomal markers (Bunnell et al., 2002). Fluorescence studies of Slp76 are described in more detail in section 5.3.5 in results chapter 5.

1.5.6.5 GADs

GADs is an adaptor protein of 322 amino acids, with a molecular weight of 38kDa, that is expressed in hematopoietic cells and most highly expressed in T cells. Its discovery was identified through yeast-two hybrid screening and also through random genomic DNA screening (Liu et al, 1998). GADS plays a key role in connecting both Slp76 and LAT as it concurrently binds to both proteins. GADS binds constitutively to SLP76 via its SH3 domains, which recognises polyproline residues (224-244) on SLP76 (Asada et al., 1000, Liu et al., 1999, Seet et al., 2007) Upon TCR ligation, GADS also binds to LAT through its SH2 domain (Lui et al., 1999) and, in addition, GADS has been reported to bind HPK-1, which is a serine/threonine kinase implicated in the activation of JNK (Ma et al., 2001).

1.5.6.6 Vav

Vav is a 95kDa protein, first discovered as an oncogene product by Katzav et al. (1989). It was identified by the isolation a constitutively active, truncated form that lacked the first 67 amino acids at its amino terminus. Truncated Vav induced oncogenic transformation of

NIH 3T3 fibroblast cell line. It was named Vav after the sixth letter of the Hebrew αbet as it was the sixth oncogenic protein that they discovered in the laboratory in Israel. Since this discovery, two additional Vav proteins have been identified so there are now three full-length mammalian Vav proteins in the family; Vav1, Vav2 (Henske et al., 1995 and Scheubel et al., 1996) and Vav3 (Movilla & Bustelo, 1999). Vav1 is expressed exclusively in haematopoietic cells whereas Vav2 and Vav3 are more ubiquitously expressed. All three are present in T cells, but Vav1 plays the most important role.

The Vav family proteins are categorised as guanine nucleotide exchange factors (GEFs) due to the ability to catalyse the exchange of bound GDP for GTP on Rac/Rho GTPases, which are involved in cytosketal rearrangements (see Figure 1.6 for how a guanine nucleotide exchange factor functions). Different Vav family proteins stimulate overlapping Rac/Rho proteins. Vav1 is a GEF for Rac1, Rac2 and RhoG and Vav2 is a GEF for RhoA, RhoB and RhoG, whereas Vav3 preferentially activates RhoA, RhoG and to a lesser extent, Rac1. Vav1 is also thought to activate Cdc42 (Tybulewicz, 2005).

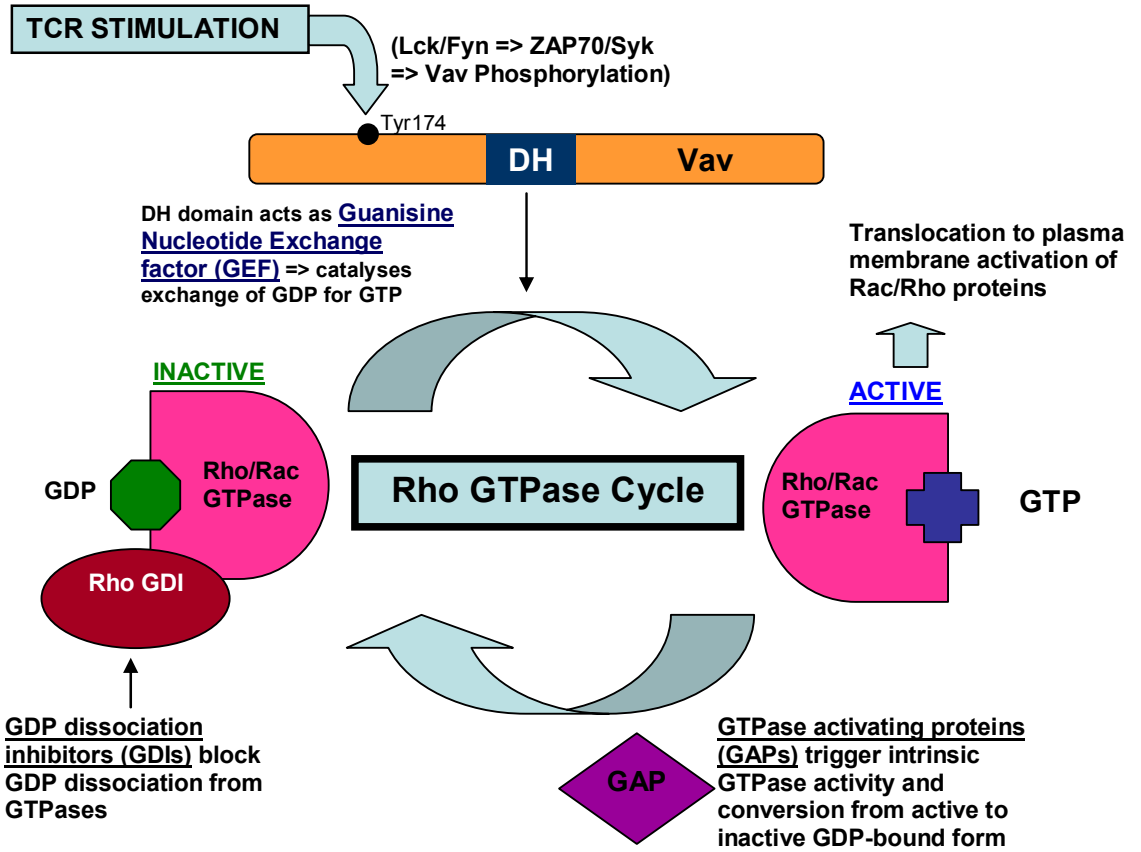


Figure 1.6 Diagram to show how Vav acts as a GEF towards Rac/Rho GTPases

Due to Vav being such a large multi-domain protein, it is connected to a sizeable assortment of binding partners. At the N-terminus lies a calponin-homology (CH) domain, which is proposed to play a role in downstream NFAT activation (Wu et al., 1995) and cooperates with calmodulin in a calcium-dependent manner to further augment the calcium flux brought about by TCR activation (Zhou et al., 2007). The CH domain also binds Ly-GDI, which regulates Rho GTPases and simulteously binds Shc (Groysman et al., 2002). It also binds to Enx-1, a polycomb family protein (Hobert et al., 1996a). Adjacent to the CH domain, the acidic (Ac) region spans 50 amino acids and contains three highly conserved tyrosine residues at positions 142, 160 and 174 that are phosphorylated during activation of

Vav (Crespo et al., 1997). Next to the Ac region lays the catalytic module, the Dbl Homology (DH) domain, which confers Vav with its GEF activity. In order to control the catalytic activation of Vav, an autoinhibitory mechanism is in operation at the N-terminus of the protein that involves the CH and Ac regions. NMR studies have revealed that unphosphorylated tyrosine 174 interacts with a pocket within the catalytic region of the DH domain, which inhibits GEF activity by preventing the DH domain-GTPase interaction. Phosphorylation of Tyr-174 disrupts this interaction, releasing an autoinhibitory loop thus restoring Vav GEF activity by allowing access of substrates to the active site. Alongside the DH domain is a pleckstrin homology (PH) domain and this domain was also found to be thermodynamically coupled to the Tyr-174 phosphorylation site through phospholipids (Aghazadeh et al, 2000). It has been proposed that binding to PIP2 is inhibitory towards GEF activity whilst PIP3 enhances it (Han et al., 1998, Das et al., 2000). As well as regulating the GEF activity of Vav, it has been suggested that the PH domain is important in membrane targeting of Vav and maintaining its locality there through its binding to phospholipid metabolism products. However, the precise role of the PH domain is still controversial and there have been conflicting reports concerning whether it helps to regulate GEF activity or not.

Next to the PH domain is a zinc finger (ZF) domain, which through mutational analyses, has been found to help stabilise the autoinhibitory interaction between the CH domain and the DH domains by interacting with the CH domain itself (Zugaza et al., 2002). At the C-terminus lies a proline-rich (PR) region, followed by an SH3-SH2-SH3 adaptor domain cassette. The N-terminal SH3 domain binds to the SH3 domain of Grb2 through a unique interaction involving the dimerisation of the two SH3 domains. This was originally

revelaed through yeast two-hybrid screening (Ye & Baltimore, 1994), but has since been confirmed in the crystal structure of the Vav N-terminal SH3 domain bound to Grb2 (Nishida et al., 2001), and through NMR studies (Ogura et al., 2002). Several tyrosine phosphorylated proteins involved in TCR signalling have been shown to interact with the SH2 domain of Vav such as Slp76 (Onodera et al., 1996, Tuosto et al., 1996), Zap70 (Katzav et al., 1994) and Syk (Deckert et al., 1996). Also proteins that negatively regulate Vav activity bind through this domain such as c-Cbl (Merengere et al., 1997) and SHP-1 (Stebbins et al., 2003). Finally, the C-terminal SH3 domain is responsible for interactions with many proteins involved in cytoskeletal reorganisation such as dynamin2 (Gomez et al., 2005) and zyxin (Hobert et al., 1996b). It also binds to RNA-binding proteins such as hnRNP-K (Hobert et al., 1994) and hnRNP-C (Bustelo et al., 1995), the transcriptional modulator Ku-70 which is part of the DNA-dependent protein kinase complex (Romero et al., 1996), the HIV-1 virus protein Nef-1 (Fackler et al., 1999) and the Kruppel-like factor VIK-1. VIK-1 is a protein that shuttles between the nucleus and cytoplasm and contains zinc finger domains thought to bind to DNA and CDK4 during cell cycle progression. Many of the proteins that bind the C-terminal SH3 domain are found in the nucleus and so it is plausible Vav enters the nucleus under certain conditions and forms a complex with these proteins. Indeed, Vav does contain two putative nuclear localisation signals (KTRELKKK and KKDKLHRR) within its sequence, residing near the C-terminus. Vav has been shown to enter the nucleus in response to certain haematopoietic receptor stimulation. For example, prolonged stimulation of the FcεRI receptor leads to Vav translocating to nuclear regions (Romero & Fischer, 1996, Houlard et al., 2002).

The role of Vav in downstream signalling pathways has been established through a combination of gene knockout studies in mice and Jurkat T cells and though mutational analyses. Vav1 knockout mice are fertile and grossly normal, but display impaired MAPK responses, calcium flux, NFAT and NFκB-dependent transcription (Costello et al., 1999). The transition from double negative to double positive T cells in T cell development is also partially blocked in Vav1-/- mice. As a result they have fewer peripheral T cells most probably due to dysfunctional pre-TCR signalling in the absence of Vav (Turner et al., 1997). A Vav knockout Jurkat cell line, J.Vav1, has been created. These cells mimic nearly all of the defects as seen in the knockout mouse model and in addition JNK MAPK activation is diminished (Cao et al., 2002). Vav is required The ERK MAPK pathway is defective in Vav deficient lymphocytes from Vav1-/- mice. This is because Vav constitutuvely binds to Grb2 through a unique binding mechanism involving SH3 domain dimerisation (Ye & Baltimore, 1994). Thus the absence of Vav may disrupt Grb2 stability and thus destabilise its interaction with Sos, which therefore would not be able to effectively initiate the Ras/Raf/MEK/ERK pathway (Reynolds et al., 2004). The absence of Vav affects activation of Ras through a different guanisine nucleotide exchange factor called RasGRP. This exchange factor does not rely upon Grb2-SOS interactions, but instead is activated through its association with the phospholipid metabolism product, DAG, which is created via hydrolysis of PIP2 by PLCγ.

In addition to its involvement in ERK MAPK signalling, Vav is also required for the actiation of the NFκB transcription factor. Vav has been implicated as a regulator of IκB kinase (IKK), which is a negative regulator of NFκB. One explanation that may account or this could be down to the influence of Vav upon PKCθ movement to the membrane where

PKCθ binds IKK leading to the release of NFκB (Villalba et al, 2000). Furthermore, Vav is also an upstream regulator of the nuclear translocation of the NFAT transcription factor. This is dependent upon the interaction of Vav with Slp76 (Raab et al., 2001). The GEF domain of Vav also contributes to NFAT stimulation through the activation of Rac, which subsequently stimulates the c-Jun N-terminal kinase (JNK), followed by enhanced transcriptional and DNA-binding abilities of AP-1 and heightened phosphorylation of c-Jun. Vav1 negative cells are defective in c-Jun phsophorylation and reconstitution with the L213A DH domain mutant of Vav fails to restore the response and still suppresses TCR-evoked c-Jun phosphorylation thereby corroborating the requirement for GEF activity (Kaminuma et al., 2001). NFAT is also activated in response to increase calcium levels and, as therefore the CH domain of Vav mediates NFAT activity also through its cooperation with calmodulin (Zhou et al., 2007). The activation of these transcription factors means that Vav is integral in the upregulation of the IL-2 gene as they bind to its promoter.

As Vav displays catalytic GEF activity towards Rac/Rho GTPases, Vav is also instrumental in many cytoskeletal processes involving filamentous actin (F-actin) and microtubules resulting from TCR perturbation. Rac activation is important in T-cell spreading, formation of lamellipodia and membrane ruffling (Bustelo, 2000). Vav is involved in clustering of lipid rafts at the immunological synapse (IS) formed upon at the T-cell/APC interface as in Vav1 deficient cells lipid rafts fail to cluster (Villalba et al., 2001). Vav is also required for the localisation of PKCθ at the IS. Vav is also essential for promoting clustering of the LFA-1 Integrin receptor at the IS and is required for the binding of integrin receptors to

their matrix ligands via "inside-out" signalling and this promotes further stability of cell-cell adhesions and cell spreading (Hornstein et al., 2004).

It has been established that Vav is deployed to the TCR and is maximally phosphorylated one minute post-ligation of the TCR (Zakaria et al., 2004). Studies have shown that the Syk family kinases, Syk (Deckert et al., 1996) or Zap70 (Margolis et al., 1992) and the Src family kinases, Lck (Han et al., 1997) and Fyn (Huang et al., 2000) are all capable of phosphorylating Vav. Within the TCR early signalling complex it is able to bind an array of proteins through its adaptor domains. The recruitment and stability of Vav is further stabilised at the TCR through the interaction of its PH domain with PIP2 and PIP3, products of phospholipid metabolism. Vav is recruited to the early signalling complex through interactions with the Grb2-SOS-Zap70 complex. In cells lacking Zap70, Vav recruitment to Grb2-containing complexes at the membrane is defective (Salojin et al., 2000). As mentioned, Vav is phosphorylated by Zap70 and the SH2 domain of Vav also physically interacts with tyrosine 315 of Zap70 (Katzav et al., 1994, Wu wt al., 1997). Vav also interacts via its SH2 domain, with Slp76 (Onodera et al., 1996). There are two phosphotyrosine residues (pYESP sites 113 and 128) that the Vav SH2 is able to bind and both of these residues rely on phosphorylation by Zap70 with no other Src or Syk family kinase able to substitute (Raab et al., 1997). One phosphotyrosine is thought to directly interact with Vav and the other is thought to stabilise the interaction (Fang & Koretzky, 1999). The TCR co-receptor, CD28, also induces a Vav-Slp76 interaction when co-ligated and the co-operation between the two molecules in response to CD28 activation serves to maximise and potentiate downstream IL-2 and IL-4 transcription by promoting nuclear

entry of NFAT (Raab et al., 2001). As well as binding Vav, Slp76 simultaneously binds GADs and another adaptor protein called Shb aids in recruiting the Slp76-Gads-Vav complex to the TCR (Lindholm et al., 2002). The Slp76-Gads complex in turn binds LAT, which indirectly links Vav to LAT. Other studies have shown that Vav also regulates activation of the Tec family kinase, Itk and also phosphorylation of PLCγ although it is still unclear whether this is due to direct or indirect interactions (Reynolds et al., 2002, Dombroski et al., 2005). The series multi-protein interactions at the TCR result in a large complex being formed and the interactions of Vav with different components help orchestrate downstream signalling pathways.

1.6 Use of the Interferon receptor to study signalling complex formation in T-cells

1.6.1 General Overview of Cytokines

It is hard to encompass all of the actions of cytokines within a single definition, however, they are generally described as pleiotrophic regulatory proteins secreted primarily from white blood cells. They contribute to a complex chemical signalling network involved in modulating both innate and adaptive immune responses, hematopoiesis, oncogenesis, neurogenesis and early embryonic development e.g. establishment of the body axis and cell polarity (Ishihara & Hirano, 2002). Cytokines act in an autocrine or paracrine fashion and the activity of an individual cytokine on a given cell relies upon the concentration of this cytokine in the cell microenvironment, the combination of other cytokines and growth factors present in the extracellular milieu, the cell type upon which the cytokine is acting

and also the duration of time that a cytokine acts upon a target cell. Environments of cells will alter depending on physiological conditions at the time e.g. normal, stressed, inflamed or tumour baring STATe of localised tissues (Ishihara & Hirano, 2002) and this can influence cytokine levels as well as the amount of receptor on cell surfaces. If multiple cytokines are present in the interstitial fluid, then the sequence in which one acts upon a target cell can also affect the response.

Cytokines can be loosely divided into two main groups depending on the type of cell that produces them. The type 1 cytokines are produced by Type 1 helper cells and the Type 2 class of cytokines are produced by Type 2 helper cells (Haddad, 2002). The type 1 class of cytokines includes interleukin (IL)-2, IFN-γ, IL-12, IL-10 and TNF-β. Examples of type 2 cytokines are IL-4, Il-5, IL-6, IL-10, IL-13, IFN-α and IFN-β. Cytokines can be further subdivided into families based upon shared secondary or tertiary structures (Haddad, 2002).

1.6.1.1 Characteristic features of cytokines (Thomson & Lotze, 2003)

(a) They are simple polypeptides or growth factors ≤ 30kDa size

(b) Their production is transient and the radius within which they act is short i.e. paracrine or autocrine as opposed to endocrine

(c) They bind their cognate receptors with high affinity

(d) The constitutive production of cytokines is normally low or absent and production is regulated by inducing stimuli

(e) Most cytokine action can be attributed to altered patterns of gene expression within target cells, which can result in cell proliferation and cell differentiation, or conversely cell cycle arrest and/or apoptotic mechanisms.

(f) At least some of the activity of an individual cytokine is directed towards cells of hematopoietic lineage

1.6.2 The Interferon Family

Interferons form one of the most important groups of cytokines that were originally described in 1957 (Isaacs & Lindenmann, 1957) as potent antiviral agents and it is because of these anti-viral and anti-proliferative attributes that they are routinely used for many clinical applications today. For example, to treat certain malignancies (Parmar & Platanias., 2003). The response of a cell to infection with double stranded RNA, which is only found within the genome of viruses but nowhere in mammalian cells, is to produce Interferons as a signal to other cells that there is a pathogenic invasion underway. Binding of secreted interferon to its receptor upon nearby cells causes cell cycle arrest and other anti-proliferative effects designed to limit the spread of the virus. It is also known that detection of bacterial ligands by a selection of Toll-like receptors also leads to secretion of Interferons (Theofilopoulos et al., 2005). Therefore, Interferons act as one of the first lines of defence against viral invasion and form an integral part of the innate immune response. It has now become clear that, as with other cytokines, their effects on target cells are much more varied and far reaching than simply just being anti viral agents as they are able to evoke multiple biological outcomes due to their regulation of hundreds of genes. As well as producing anti-proliferative effects, they are also important in bringing about anti-tumour

effects, anti-angiogenic effects as well as exhibiting immunomodulatory activities (Parmar & Platanias, 2003). It is thought that they help to aid migration of immune cells by acting as a chemotactic cue. Also IFNα, since it is one of the first cytokines to be produced by the immune system in response to viral infection, may "prime" surrounding cells at the site of infection so that non-infected cells are prepared, presumably by induction of cell cycle arrest. It has been shown that low doses of interferon administered to cells prior to viral infection result in increased protection (Beilharz et al., 1997). Interferon effects differ depending on cell type and also may synergise with other cytokines to modify and fine tune the precise cellular response. In addition, the action of interferons upon some cell lines is not solely anti-proliferative and infact mitogenic pathways can be induced. This seems to depend on the cell type, the length of time interferon stimulates a cell receptor as well as the activation STATs of a cell and the other cytokines present. For instance, IFNα increases $CD8^+$ T cell proliferation. I will discuss this in more detail later.

The interferon family are broadly categorised as belonging to the Type II cytokine family based on shared similarities of the structure of the receptor that they bind with other members of this group, which always has tandem fibronectin type III domains. The complete family of type II cytokines is reviewed in Kotenko & Pestka, 2000. All Interferons (IFNs), like other cytokines, bind to receptors that lack an intrinsic tyrosine kinase domain. Instead they associate with cytoplasmic protein tyrosine kinases, mostly of the JAK family of kinases. In the 1980s, IFNs were sub-divided into two groups based not only on the cell type responsible for their production but also based on the multi-chain

receptor through which they bind to at the cell surface (Aguet et al., 1984, Merlin et al., 1985). The two families of IFNs are known as the Type I IFNs and Type II IFNs.

The later group only consists of one member called IFN-γ, first discovered in 1965 by Wheelock et al. described as a virus-inhibiting protein. IFN-γ is secreted as a homodimer by T-cells and natural killer (NK) cells and binds to a unique receptor known as the type II interferon receptor, which is composed of two subunits named IFNGR1 and IFNGR2 (Schlindler et al., 1995). IFNGR1 is constitutively associated with the JAK1 kinase whereas the IFNGR2 subunit constitutively associates with JAK2 kinase (Bach et al., 1997).

However, the type I interferon group is a larger family and includes IFNα, β, ω, κ and ε. Only one single type of β, ω, κ and ε IFNs exists but the IFN-α family comprises 13 subtypes (Pestka, 1987). Type I IFNs can be produced by virtually all cells in response to pathogens at low levels, but it is the plasmacytoid dendritic cells (pDCs), a type of antigen presenting cell, that are the most potent producers of IFN-α/β as they have the capacity to produce 1000-fold more than other cells of the body (Siegal et al., 1999). The reason that these cells produce higher levels than other receptors is that they have a different profile of microbial pattern recognition Toll-like receptors as they preferentially express TLRs 7 and 9, which allows them to respond to single stranded RNA and DNA viruses respectively (reviewed in Asselin-Paturel & Trichieri, 2005).

The entire family of class one cytokines signal through the same common receptor known as the type one interferon receptor or Interferon α receptor (IFNAR), which is a multi-subunit structure composed of two chains, IFNAR1 (Uzé et al., 1990) and IFNAR2 (Novick et al., 1994, Domanski et al., 1995, Lutfalla et al., 1995). IFNAR1 has a large N-

glycosylated ectodomain, with two cytokine binding modules, and intracellular region of 100 residues (Ling et al., 1995). It has four tyrosine residues within the intracellular region at positions 466, 481, 527 and 538, but only 466 and 481 have been shown to be Tyk-2 phosphorylated and the other residues have been suggested to be necessary for down-negative regulation of the receptor as their deletion enhances IFNAR signalling (Gibbs et al., 1996). Two different forms of IFNAR2 exist due to differential splicing, which are called IFNAR2b (Novick et al., 1994) and IFNAR2c (Domanski et al., 1995, Lutfalla et al., 1995). It is only the IFNAR2c splice variant, which harbours a long cytoplasmic tail that is expressed as part of the functional IFN receptor complex (Lutfalla et al., 1995). IFNAR2c encompasses five proximal tyrosines at positions 269, 306, 316, 318 and 336. Following ligation of the IFNAR with a class one IFN, each of the subunits interacts with a member of the JAK kinase family. IFNAR1 is constitutively associated with Tyk2, another member of the JAK family of kinases, whereas IFNAR2 is constitutively associated with JAK1 (Colamonici et al., 1994a, Domanski et al., 1995).

In response to an IFN ligand the subunits of both the type I and type II receptors dimerise, very much like RTKs, and auto- and trans-phosphorylation and activation of the associated JAK kinases ensues. Phosphorylated JAK kinases subsequently regulate the phosphorylation of signal transducer and transactivator (STAT) proteins and the resulting JAK-STAT pathway. STATs can form homo- or hetero-dimers with other STAT proteins and translocate to the nucleus where they bind to specific gene promoter sequences. Many of the signalling events just mentioned were established in the laboratories of Jim Darnell, Ian Kerr and George R. Stark, as discussed in more detail in section 1.6.3.1.

The JAK-STAT is the best characterised signalling pathway originated by IFNs, but more recently it is becoming increasingly clear that this cascade alone is not sufficient to generate all of the biological effects of interferons upon the cell. Thus, other cascades and signalling proteins must be involved in initiating the correct downstream gene transcription for a specific response (Platanias, 2005). Therefore, assortments of non-JAK/STAT proteins form a multimeric complex at the activated IFNAR in order to manipulate the downstream response. In the following results chapters the Type I family of Interferons are the focussed upon, more specifically IFNα because the receptor is expressed on the T-cells surface. Also IFNα has been used to treat a variety of clinical conditions so a pure form of the ligand is readily available for use (see section 1.6.6 for more detail). The sub-type of IFNα used for clinical treatment of malignancies and certain viruses is IFNα2, which is the subtype that is used for all experiments.

1.6.3 Signalling pathways emanating from the Interferon α receptor, expressed on the T-cell surface

The precise nature of the extracellular signals is transmitted to the nucleus through activation of a variety of essential protein cascades initiated by an assortment of proteins recruited to complexes formed at the activated receptor. Initially the JAK-STAT pathway was emphasised as controlling downstream events but it is becoming increasingly clear that in order to regulate hundred of genes, other non-STAT and MAPK cascades are needed too, which are less well defined at present. The STAT, non-STAT and MAPK cascades are shown on Figure 1.8 and are described in more detail in the following sections.

1.6.3.1 JAK-STAT signalling pathway

This signalling cascade was first discovered through studies of the Interferon Receptor and results in many of the antiviral and growth inhibitory effects induced by the IFNAR (Pestka, 1981a, Pestka 1981b, Pestka, 1986, Pestka et al., 1987). It is now clear that all type 1 and type 2 cytokine signalling requires signal transduction through the JAK/STAT pathway and it is not a pathway exclusive to IFN receptors.

Much of the early work to elucidate signalling events at the IFNAR, as well as how JAK/STAT proteins are recruited and activated in response to Interferon α, was carried out using two parallel and complementary approaches: firstly biochemistry and gene cloning, and secondly somatic cell genetics (Darnell et al., 1994).

In the laboratory of Jim Darnell biochemical and gene cloning methods were utilised. Cells were stimulated with IFN-α or γ and cDNAs complementary to mRNAs that were upregulated were isolated. Isolation of these genes allowed the identification of a common site known as the Interferon stimulated response element, which was found to be a highly conserved element in the 5' flanking promoter-enhancer region of these genes (Levy et al., 1988, Porter et al., 1988). Initially a complex known as factor E was found to translocate from the cytoplasm to the nucleus bind to the promoters of ISGs and stimulate ISRE-dependent transcription (Dale et al., 1989). This element was later renamed ISGF3 and the components were subsequently identified and cloned. They were named STAT1, STAT2 and IRF9 (Fu et al., 1992, Schindler et al., 1992). Another element found to be at the promoter of some genes is the IFN-γ activation site (Darnell et al., 1994).

The somatic cell genetic approach was largely carried out in the laboratory of G.R. Stark, where chemical mutagenesis experiments resulted in a number of different cell lines

lacking components required for Interferon α and γ signalling. Such approaches lead to the identification of the JAK family kinase, Tyk2 using the U1A cell line (Velazquez et al., 1992). These cell lines were also used to understand how STATs1 and 2 were activated and identify other Jak family members (Shuai et al., 1993).

The JAK kinase family has now been found to comprise four members; JAK1, JAK2, JAK3 and Tyk2. The JAK family lack any SH2 or SH3, but instead posses a variety of well-conserved JAK Homology (JH) domains of which they all have seven (JH1-7). Of these JH1 and JH2 have extensive homology to the tyrosine kinase domains although JH1 seems to be the only functional kinase domain whereas JH2 just seems to be a kinase-like domain (Rane & Reddy, 2002). The roles of the other domains are still under investigation but it has been shown that, in the case of Tyk2, the JH3-5 are required for stabilising binding to the IFNAR (Richter et al., 1998). For JAK1, the other domains are also known to stabilise binding at the receptor and truncation experiments showed that the first 166 amino acids in the JH6 and JH7 domains are imperative for efficient binding to IFNAR1 and their absence prevents JAK1 activation. A region that lies within JH3-5 is also required for stable binding (Usacheva et al., 2002).

The type I Interferon receptors utilise only JAK1 and Tyk2 for signalling (Kalvakolanu et al., 2003). Ligand binding of α/β IFN induces dimerisation of the receptor which leads to trans- and autophosphorylation of the associated JAK kinases. Phosphorylation of JAK1, which is constitutively associated with IFNAR2, results in intracellular phosphorylation of the opposite cytoplasmic chain of the receptor through trans-phosphorylation i.e. IFNAR1 (Fu, 1992, Schindler et al., 1992, Gauzzi et al., 1996) and phosphorylation of Tyk2,

associated with IFNAR1 results in phosphorylarion of tyrosine residues on IFNAR2 (Colamonici, 1994b).

This reciprocal phosphorylation of the receptor by JAK kinases creates docking sites for the SH2 domains of STAT proteins. These are a group of latent cytoplasmic transcription factors, which are elementary in mediating downstream gene expression (Darnell et al., 1994, Darnell, 1997). Once recruited to the cytoplasmic tail of the IFNAR1 or 2, STAT proteins undergo tyrosine phosphorylation on a specific residue located in their C-terminal region. This modification causes release from the receptor into the cytoplasm, where they then bind other activated STAT proteins to form homo- or hetero-dimers. This interaction involves the tyrosine phosphorylated residue on one STAT protein binding to the SH2 domain of another STAT. There are seven members of the STAT family of proteins; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 (Caraglia et al., 2004). Of these STATs 1, 2 ,3, 5a and STAT5b have all been identified as substrates for the IFN-α receptor within the T-cell. STAT4 and STAT6 are both expressed in T-cells, but they are activated in response to other cytokine receptors and are not involved in IFN-α signalling. However, STATs 4 and 6 can be activated by IFNα in other cell types e.g. epithelial cells (Platanias, 2005). Once dimerised, STATs immediately translocate to the nucleus where they are able to bind distinct transcription elements (Sadowski et al., 1993) and, in concert with other nuclear proteins, help initiate transcription of a diverse array of genes. See figure 1.7 for a diagram of possible STAT dimers that can form.

1.6.3.1.1 STAT 1 and STAT2

STATs 1 and 2 are responsible for many of the anti-proliferative effects seen in response to IFNα such as cell cycle arrest and induction of pro-apoptotic genes. STAT1 can form either a homodimer or a heterodimer with STAT2 (Li et al., 1996). This occurs through recruitment of STAT2 phosphotyrosine docking sites at positions 466 and 481 of IFNAR1, created by activated Tyk2 (Krishnan et al., 1998). STAT2 is then phosphorylated at position 690 and generates a binding site for STAT1 (Fu, 1992, Fu et al., 1992). STAT1 is then, in turn phosphorylated by JAK1 (Leung et al., 1995).

The tyrosine phosphorylated STAT1/2 heterodimer can then bind to IRF-9 (also known as p48) to form a trimeric complex called ISGF-3 (Improta et al., 1994). This complex then translocates to the nucleus and can then bind to the consensus sequence AGTTTCNNTTTCC (where N is any nucleotide) known as the interferon stimulated response element (ISRE) present in Interferon responsive genes (Darnell et al., 1994). Alternatively the STAT1 homodimer can bind IFN-γ activated sites known as GAS elements (Decker et al., 1997), which are also present in the promoters of Interferon-stimulated genes (ISGs). Some genes contain both ISREs and GAS elements whereas other ISGs contain just one or the other. Some of these genes are described in section 1.6.5. In addition to tyrosine phosphorylation, C-terminal serine phosphorylation of STAT1 on position 727 occurs and this is thought to be regulated either the PI3K pathway, PKC-δ or ERK1/2 (Nair et al., 2002, Gamero & Larner, 2000). Serine phosphorylation of STAT1 is not required for nuclear translocation or binding to promoters, but is required for full transcriptional activation (Platanias, 2005).

Using knockout mice studies it has become evident that ablation of the STAT1 gene enhances mitogenic effects induced upon IFNα stimulation. Cells stimulated with IFNα from STAT1-/- mice proliferate rather than arrest of the cell cycle. They exhibit reduced STAT5 phosphorylation levels, but STAT2 and STAT3 are both highly phosphorylated. Similarly IFNα-stimulated STAT2-/- cells taken from knockout mice also proliferate rather than arrest. This highlights the importance of the ISGF-3 complex in mediating the anti-proliferative effects normally seen in IFNα stimulated cells (Gimeno et al., 2005). STAT3, which is discussed in more detail in the following, section has been thought to be crucial for survival pathways e.g. involving PI3-K/Akt so it could have been the case that it the absence of STAT1 or STAT2, the signals augmented by STAT3 could have dominated leading to the marked increase in proliferation of T cells upon IFNα treatment. However, knocking out the STAT3 gene through the Cre-recombinase method in STAT1 knockout mice did not halt the proliferative effects seen in the absence of STAT1 as STAT3 absence only had a marginal effect on the mitogenic response of STAT1-deficient cells (Gimeno et al., 2005). Therefore, the proliferative response seen in the absence of STAT1 or STAT2 is not purely due to survival signals mediated by STAT3.

1.6.3.1.2 STAT3 and STAT5

The other STAT proteins involved in IFNα signalling in T cells are STAT3, STAT5a and STAT5b. These STATs can form STAT1:STAT3, STAT3:STAT3, STAT5:STAT5 and CrkL:STAT5 homo or heterodimers (Figure 1.7) in order to control transcription of additional sets of interferon regulated genes. The significance of the STAT5:CRKL heterodimer is referred to in section 1.6.4.3.1. Among the seven known STAT proteins,

STAT3 is unique because only the STAT3-null phenotype in mice results in an embryonic-lethal phenotype at day 6-7 of fetal development, which cannot be compensated by any of the other STAT species (Akira, 1999).

Piceatannol, a Syk-kinase, inhibitor is able to halt STAT3 and STAT5 phosphorylation since it inhibits Tyk2 due to its high homology with Syk (Su & David, 2000). Tyk2 is still phosphorylated due to intact JAK1 activity in the presence of piceatannol, but JAK1 is not phosphorylated as it relies upon transphosphorylation by Tyk-2, whose catalytic ability was abolished by the inhibitor. Residues upon IFNAR2 were not phosphorylated either due to Tyk2 being inactive. This shows that STAT3 and STAT5 are recruited to IFNAR2 phospho-tyrosines where they rely upon activated Tyk-2 for phosphorylation (Su & David, 2000).

In addition, like STAT1, complete STAT3 activation requires not only tyrosine phosphorylation at position 705, but also requires serine phosphorylation on serine 727 (Wen et al., 1995) and one of the proteins which is thought to do this is activated ERK MAPK (Chung et al., 1997).

STAT3 has been shown to be phosphorylated not only upon IFNα stimulation, but also upon stimulation with an anti-CD3 antibody, which activates the TCR, and this phosphorylation occurs within a minute of antibody stimulation and can still be detected for up to 60 minutes (Gerwein et al., 1996). This relies upon a Src kinase e.g. Lck or Fyn, since PPI, a Src-kinase inhibitor, abolishes STAT3 phosphorylation upon TCR stimulation (Gerwein et al., 1996). Src family induced phosphorylation of STAT3 could represent a point of cross-talk between the TCR and cytokine induced receptors.

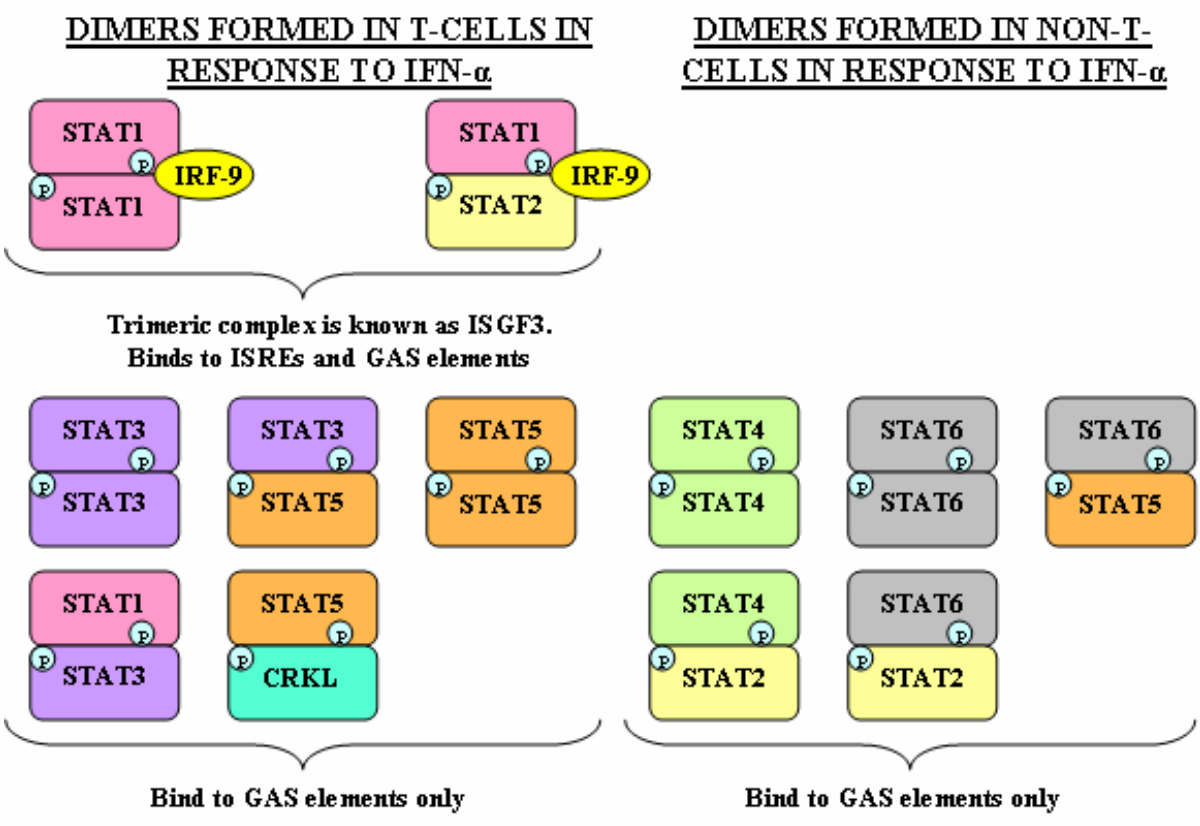


Figure 1.7 Diagram to show the STAT dimers formed in T cells and non T-cells

1.6.4.2 Non-STAT Signalling through MAPK Pathways

IFNAR ligation has been shown to bring about phosphorylation of ERK1/2 as well as p38 MAPK associated with stress responses. The p38 family of MAPKs includes several isoforms; p38α, p38β, p38γ and p38δ, which are all encoded for by distinct genes although they share high structural homology. The p38 MAPK pathway is a stress pathway and is essential in the anti-viral effects of IFN (reviewed in Platanias, 2003). Phosphorylated p38 can activate an array of downstream proteins such as MAPK-activated protein kinases 2 and 3 (MAPKAPK2/3), which are required for transcription of proteins such as Isg15. Also p38 is thought to activate Mitogen and stress activated kinase1 (MSK1) and MSK2, which are required for histone acetylation to relax the DNA structure and allow gene transcription

(Platanias, 2005). There is no evidence that the p38 pathway relies upon STAT activation but there is evidence that it is activated downstream of the G-protein Rac1 because a dominant negative Rac1 mutant blocks IFN-dependent p38 activation (Uddin et al., 2003). Rac1 is activated by the GEF activity of Vav1, which is activated in response IFNα/β. This therefore implicates Vav in downstream p38 signalling.

The role of ERK1/2 activity induced through IFNAR ligation is still uncertain but at other receptors, this pathway is utilised in order to exert cellular growth and differentiation (see sections 1.3.1.1 and 1.3.1.2 for explanation of ERK signalling). One study showed that ERK2 is required for serine phosphorylation of STAT1, which needed for full activation of this protein (Zhang et al., 1995). This suggests a role for ERK2 in mediating a role in control of complexes binding to both ISRE and GAS elements. The MEK/ERK pathway is activated very shortly and transiently after IFNα stimulation but is reduced back to basal levels when cells are stimulated 30 minutes or longer (Arora et al., 1999, Ahmed et al, 2005). ERK1/2 phosphorylation can be strongly reduced upon long term treatment of IFNα. 48 hour treatment of Jurkat cells with IFNα drastically reduces MEK phosphorylation at Serines 217 and 221, but the activity of Ras and Raf-1 is unchanged (Riva & Zella, 2000). Longer term treatment also prevents CDK inhibitors such as p21/Waf-1/CIP-1 or p27 being degraded therefore preventing cell cycle progression (Romerio & Zella, 2002). Culturing Jurkat cells in low amounts of IFNα leads to a profound reduction in cellular proliferation by six days, which also proves the anti-proliferative long-term effects of IFN-α (Romerio et al., 2000).

The function of the short-term ERK1/2 activation of cells is not yet established, but it seems that the length of stimulation with IFNα is critical for determining downstream

effects. Short-term MAPK activation also relies upon Lck (Lund et al., 1999) and Zap70 (Ahmed et al., 2005) since their absence impedes the transient activation of ERK1/2. A possible function of the activation of ERK1/2 could be to serine phosphorylate STAT1 as STAT1 contains an XPSXP MAP kinase motif in the C-terminus, in which a serine residue is present. Studies have shown that this residue in STAT1 is required for full transactivating abilities (Kalvakolanu, 2003). One report deduced that ERK2 actually associates with the IFNα receptor before serine phosphorylating STAT1 (David et al., 1995). Another function of ERK cascades in IFN-mediated signalling is the regulation of IFNγ-dependent transcription by CCAAT/enhancer-binding protein-β (C/EBP-β) which is a transcription factor that binds response elements known as GATEs, IFN-γ-activated transcriptional elements (Platanias 2005). C/EBPs transcription factors belong to a superfamily, which includes CREB, Foc, Jun/AP-1, ATF and Maf/Nrf (Kalvakolanu, 2003). It has been shown that MEK inhibitors block IFN-induced transcription at GATEs and the same effect is seen when cells are transfected with dominant-negative ERK1/2 mutants (Kalvakolanu, 2003). The importance of ERK1/2 in C/EBP-β activation rings true for γ-interferon, but it is not yet known whether IFNα induces this transcription factor through ERK1/2 signalling also.

1.6.4.3 Other non-STAT pathways

1.6.4.3.1 Crk pathway

The CRK family family of proteins act as adaptors as they contain SH2 and SH3 domains. There are three CRK family proteins, CRKL, CRKI and CRKII, which are all cellular

homologues of viral CRK (Platanias, 2005). Of these proteins CRKL and CRKII are required for the growth inhibitory effects of IFNα and IFNγ. CRKL is tyrosine phosphorylated by Tyk2 at the activated IFNR. CRKL is then able to bind C3G, which is a GEF for Rap1, a small GTPase that is related to Ras. Rap1 antagonises the Ras pathway and thus mediates suppression of cell growth (Platanias, 2005). Furthermore, CRKL is capable of forming a heterodimer with STAT5, which can translocate to the nucleus and bind to the palindromic sequence, TTCTAGGAA, present in the promoters of some ISGs (Parmar et al., 2003). CRKL knockout mice studies have shown that CRKL:STAT5 heterodimers are of importance for transcription of genes with GAS elements (Lekmine et al., 2002).

1.6.4.3.2 IRS/PI3K

Type I interferons are also capable of bringing about Insulin Receptor Substrate (IRS) signalling, which is a signalling pathway more typically associated with the Insulin Receptor. JAK1 is capable of tyrosine phosphorylating both IRS-I and IRS-II, which serve as multi-site docking proteins with various sites that are bound by the SH2 domains of other proteins. IRSI and II regulate downstream PI3-K signalling. The p85 regulatory subunit of PI3-K is able to bind IRSI through both its N- and C-terminal SH2 domains. This association also provides a link between the IFNR with the FRAP/MTOR protein, which is activated downstream of PI3K and subsequently controls phosphorylation of the p70 S6 kinase which is a serine/threonine kinase that regulates phosphophorylation of the S6 ribosomal protein (Lekamine et al., 2003). MTOR can also phosphorylate the a repressor of translation protein called 4EBP1.

PI3-K is also capable of regulating phosphorylation of the pro-survival, anti-apoptotic protein Akt (also known as PKB). This pathway is thought to act in a cell restricted manner and depend on the precise nature of the stimulus in order to mediate survival of cells in response to IFN. Recently, it has been found that PKC-θ is also activated in a PI3-K dependent manner, which in turn is able to regulate MKK4, a protein upstream of the p38 and JNK MAP kinases, as well as being able to regulate to regulate transcription of certain ISGs containing GAS elements within their promoters (Srivastava et al., 2004).

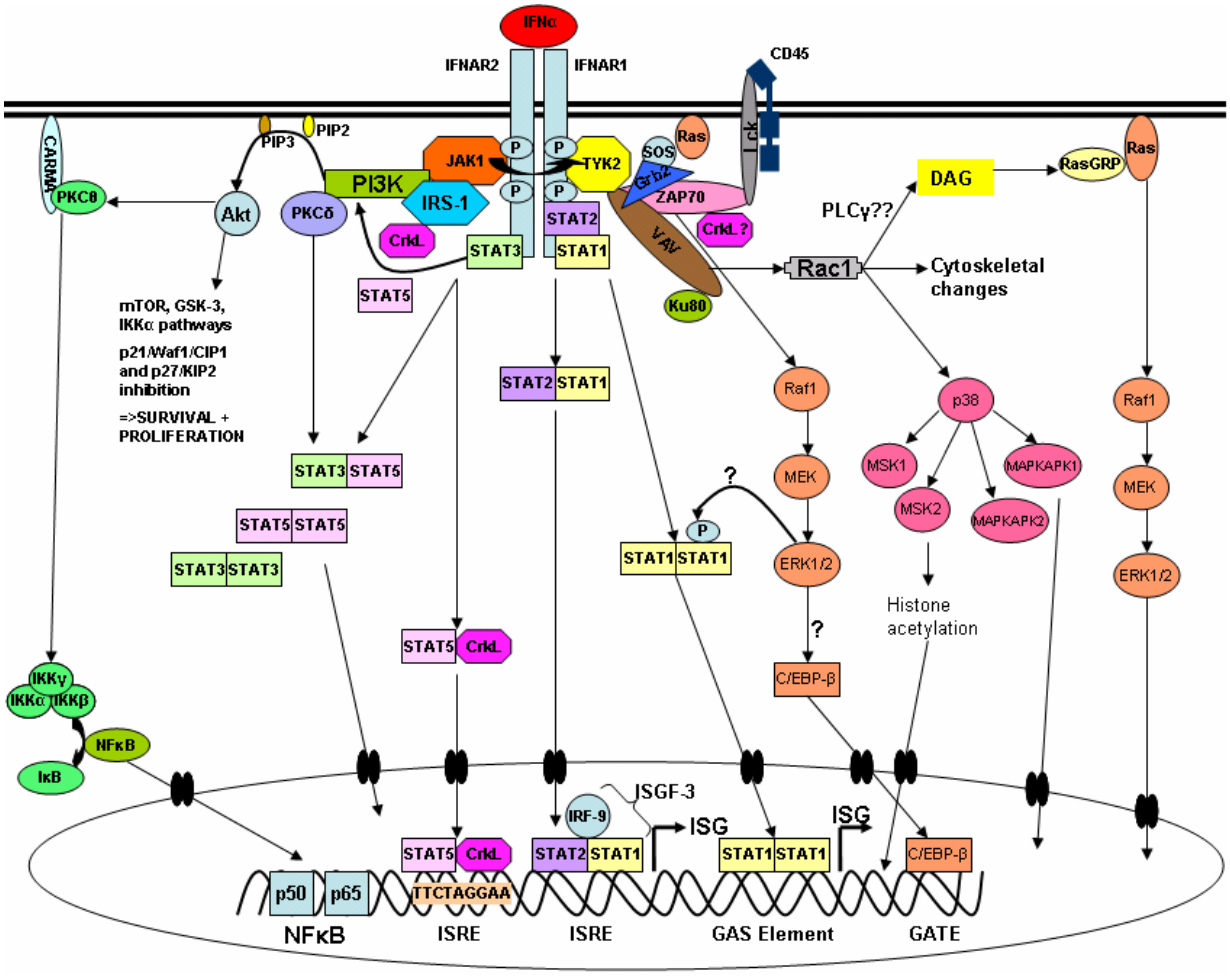


Figure 1.8 IFNAR Signalling

IFNα binding to the homodimeric IFNAR results in phosphorylation of the chains by the associated NRTKs, JAK1 and Tyk2. This results in recruitment and phosphorylation of STAT proteins that dimerise and translocate to the nucleus in order to mediate transcriptional changes Other non-STAT pathways are also activated e.g. ERK MAPK, PI3K/Akt and the NFκB pathway. I have shown on the diagram possible

1.6.5 Summary of modular organisation of key adaptors and enzymes involved in JAK/STAT signalling induced by the IFNAR in T-cells

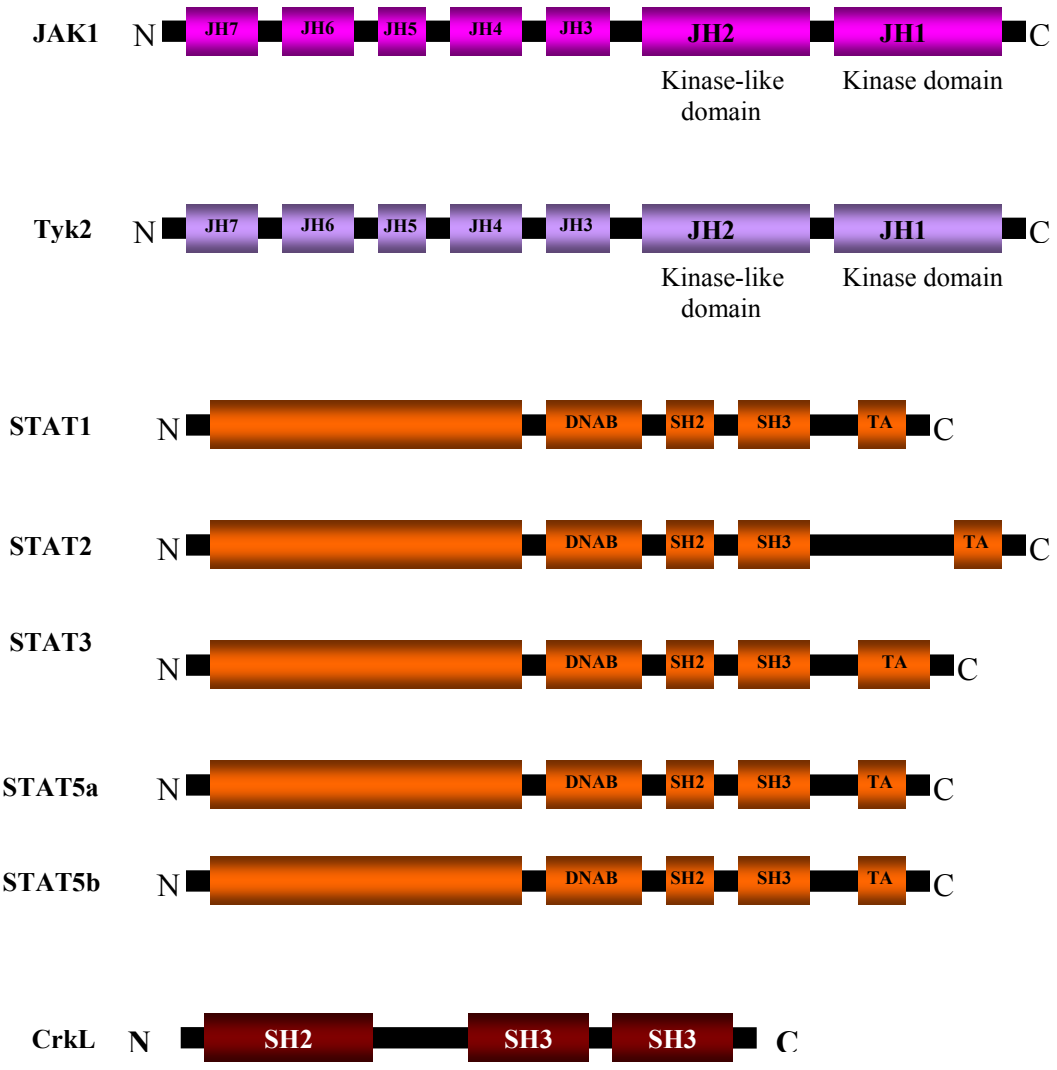


Figure 1.9 Modular Organisation of proteins utilised in IFNAR Signalling for JAK/STAT Signalling

KEY: JH = JAK Homology; DNAB = DNA binding; SH2 = Src homolgy2; SH3 = Src homology 3

Figure 1.9 shows some of the key proteins involved in IFNAR signalling. This includes JAK proteins, which consist of a series of JAK homology domains (JH). Only one of these domains is a functional kinase domain (JH1). JH2 is kinase-like but lacks functionality.

The other domains help to stabilise binding at the receptor. JAK1 associates constitutively with the IFNAR2. Also shown is Tyk2, which constitutively associated with IFNAR1 and, like JAK1, it comprises 7 JH domains, with JH1 being the kinase domain. Additionally, other domains are also required for stable binding. JH6-7 are mandatory in stable binding to IFNAR1 and also an unspecified region that lies within JH3-5 is also required (Richter et al., 1998). The association of Tyk2 with IFNAR1 aids proper receptor expression as it stabilises this receptor subunit at the cell surface and without it IFNAR1 accumulates in endocytic vesicles due to constitutive degradation (Ragimbeau et al., 2003). Tyk2 also helps to negatively regulate the IFNAR following ligation as it serine phosphorylates Ser-535, which in turn leads to recruitment of the E3 ubiquitin ligase, -TrCP2, leading to ubiquitation and proteolysis (Marijanovic et al., 2006).

Lastly**,** STAT proteins are depicted which range from approx 750-850 amino acids in length. They possess a putative SH3 domain, an SH2 domain that allows the proteins to dimerise with other STAT proteins, a DNA binding domain (DNAB) and a transactivation (TA) domain that contains a conserved phosphorylatable serine residue required for full activation. The C-terminal also harbours a conserved phosphorylatable tyrosine residue, also essential for dimerisation,

1.6.6 Genes stimulated by the IFNAR

JAK/STAT and non-STAT pathways serve to regulate the formation of transcriptional complexes such as the IRF9 complex form at the promoters of ISGs, which result in effects listed in Figure 1.10.

Response	Gene	Function
Antiviral	Mx	Viral replication
	2'5'OAS	Induces mRNA degradation
	RNaseL	Involved in the IFN-regulated 2-5A system
	PKR	Inhibition of RNA translation by phosphorylation of eIF-2
Antiproliferative	p15	Inhibits cell cycle progression
	Cyclin D	Regulation of cell cycle progression
	PKR	Inhibits cell growth
	p21,p27	Inhibits cell cycle progression
	pRb	Necessary for cell cycle G1/S transition
	c-Myc	Activation of genes involved in cell cycle G1/S transition
Apoptotic	Bax, Bak	Facilitate depolarisation of the mitochondrial membrane
	IRF-1	Induction of apoptosis in response to DNA damage
	Caspase-1	Induction of apoptosis by introducing production of IL-1β and IL-18 in macrophages
	DAPs	Mediator of apoptosis by a relatively undefined mechanism
	PKR	Mediates TNF-α induced apoptosis in response to viral infection
	Fas/FasL	Induction of extrinsic apoptotic pathway through death receptor binding
	TNF-α	Induction of apoptosis through the TNF receptor
Immunomodulatory	Tap-1, Tap-2	Transporter associated with antigen presentation
	MHC class I	Immunosurveillance by cytotoxic CD8+ T cells

Figure 1.10 Genes which are induced by type I Interferons and the effects that the gene products have upon the cell (adapted from Maher et al., 2007)

1.6.7 Clinical applications of IFNα

Interferons were first discovered as antiviral compounds (Isaacs & Lindenmann, 1957), but since then their anti-tumour and immunoregulatory properties have been recognised

(Gresser et al., 1969, Lindahl et al., 1976). Due to their abilities to halt tumour growth, the therapeutic potential of IFNs was quickly recognised. They have now been used in 40 countries in the treatment of more than 14 types of cancer. This includes haematological malignancies such as hairy cell leukaemia (HCL), chronic myeloid leukaemia (CML) and some B- and T-cell lymphomas as well as treatment of certain solid tumours, such as melanoma, renal carcinoma and Kaposi's sarcoma (Ferrentini et al., 2007). IFNα2 was first used to treat HCL in 1986, but as many patients relapse, other drugs have now taken a president. For CML, in the 1980s it was found that combination therapy of a low dose of IFNα with another drug called cytrabine has proven most effective (Jonasch & Haluska, 2001). Due to clinical advances, the use of IFNs has now been replaced in some cases with other anti-cancer drugs e.g. for the treatment of CML or HCL. They are still used for some viral diseases and melanomas and, increasingly, Interferons are used in combination with other therapies.

The pleiotrophy of IFNα means that its effects as a drug will be individual to each condition and these effects are much more intricate and multifaceted than previously realised. A combination of direct anti-proliferative effects and indirect effects through immune system modulation will be triggered. IFNα promotes increased activity and proliferation of cytotoxic T-cells and NK cells. It also promotes upregulation of MHC I expression, resulting in increased antigen presentation and increased immunosurveillance and cognate cell-mediated cell killing to eliminate virally infected cells. Tumour cells exhibit a partial or complete loss of MHC I complexes and therefore increased immunosurveillance may help detect this. In addition, IFNα can act by sensitising cells for apoptosis by upregulating extrinsic apoptotic receptors such as the Fas ligand receptor.

IFNα also upregulates proteins associated with cell cycle arrest (Brassard et al., 2002). A combination of all of these factors and the ability of IFNα to regulate so many ISGs that lead to the secretion of cytokines and upregulation or downregulation of receptors as well as many other cellular changes regulation of other genes and receptors makes IFNα with its Although the use of Interferons in medicine is invaluable in some circumstances, administration is associated with a number of toxicity-induced side effects, which depend on dose and time course of treatment. The side effects range from less severe effects such as mood disorders, fever and fatigue to more severe effects such as anaemia, thyroid and endocrine dysfunctions and so must these effects must be managed (Vilcek, 2006). In some cases alternative drugs are now used with less severe effects. There are various preparations of different Interferon subtypes produced through recombinant DNA technology that are commercially available and used therapeutically. I will be using Roferon, a commercially available IFN-α2, normally used as a drug.

1.7. The T-cell receptor and Interferon α receptor require overlapping subsets of proteins within the early signalling complex

Although the TCR elicits cell growth and the IFNAR elicits growth inhibitory effects, the two receptors are now known to utilise a common set of proteins in order to mediate this opposing outcomes. Petricoin III et al., (1997) originally showed that signalling through the IFNαR requires proteins also utilised by the T-cell receptor, through the use of peripheral blood lymphocytes (PBLs). Immunoprecipitation of IFNAR1 showed that Lck, Zap70 and

CD45 all rapidly associate with this chain of the receptor upon ligation. Jurkat cell lines lacking expression of Zap70, Lck or CD45 (the P116, J.Cam1 and J45.01 cell lines respectively), were insensitive to the inhibitory effects of IFNα administration after 72 hours, whereas IFNα inhibited growth in parental Jurkat cells by 40-50% after 72 hours. The inhibitory effects of IFNα could be restored upon reconstitution of the J.Cam1, P116 or J45.01 cell lines. Additionally, Zap70 becomes phosphorylated upon IFNR stimulation and this is reliant upon the same sequence of events seen in response to TCR ligation. In support of this, Zap70 phosphorylation or association with the IFNAR was not observed in response to IFNα in cells lacking either CD45 or Lck. Lastly, it was demonstrated that the defects shown by CD45, Lck or Zap70 negative cell lines occur independently of JAK/STAT signalling as tyrosine phosphorylated STAT1 and STAT2 were able to bind ISREs and form transcriptional complexes at a comparable level to wild-type Jurkat cells.

Since these initial experiments, other reports have not only corroborated roles for these proteins at the IFNAR, but have also exposed that the pathway for which they are required is the ERK MAPK pathway, which is a non-STAT pathway. Lund et al. first demonstrated a role for Lck in activation of downstream ERK MAPK in 1999. ERK activation was observed in J.Cam1 cells that lacks Lck expression. It was ascertained that STAT1 and STAT3 phosphorylation was still intact in the presence and absence of Lck therefore MAPK activity must be affected by a pathway independent of these STAT proteins. Since then, the time course of ERK1/2 phosphorylation has been quantified in Jurkat T cells and some of the proteins involved in this cascade have been confirmed (Ahmed et al., 2005). The heightened level of ERK1/2 phosphorylation in response to IFNα is much more

succinct than that seen at the TCR. ERK1/2 phosphorylation persists in excess of an hour upon TCR ligation, but upon IFNAR-induced ERK1/2 phosphorylation returns to basal levels after 30 minutes (Ahmed et al., 2005).

Previous work by Ahmed et al., (2005) in this laboratory has shown that Zap70 is also required for MAPK activation by the IFNAR and that mutation of the tyrosine at position 319 of ZAP70 eliminates this downstream MAPK response. Co-localisation and FRET experiments using fluorescently tagged constructs revealed that Zap70 and Lck co-localise and physically associate in response to IFNα but this association is halted in Zap70 deficient cells (P116 cells) reconstituted with Y319F mutated Zap70 showing the importance of this residue. Also Zap70 and Lck were shown to translocate to nuclear compartments after 5 minutes of IFNα stimulation. This further demonstrates a complex formation similar to that seen at the TCR.

Vav is also activated in response to IFN class one cytokines also, including α, β and ω (Platanias & Sweet, 1994). In the U-266 human myeloma cell line, upon IFNAR stimulation, Vav1 translocates from the nucleus to cytosolic fractions where it associates with TYK-2 (Uddin et al., 1997, Adam et al., 2000). Vav also interacts with Ku-80 and it is able to activate JNK and Rac-dependent pathways (Adam et al., 2000). However, whether Vav plays a similar role in T cells or whether it also plays a role in downstream MAPK activation remains to be assessed. Other studies have established a role for the p85 and p110 subunits of PI3-K. The p85 subunit associates with IRS-1 in response to IFNα and the p110 subunit is required for IRS-1 phosphorylation (Uddin et al., 1997b). PI3-K is also activated in response to the TCR and so this study draws yet another parallel between the two receptors. Wortmannin, an inhibitor of PI3-K, resulted in partial inhibition of

downstream MAPK activity suggesting that PI3-K is required upstream of this cascade. Wortmannin did not however affect formation of the ISGF3 DNA binding complex, which comprises phosphorylated STAT1 and STAT2 nor did it affect the expression of the ISG-15 Interferon-stimulated gene, which is dependent on the JAK/STAT pathway (Uddin et al., 1997b). These data suggest that PI3K, like Zap70 and Lck may function in ERK MAPK signalling independently of JAK-STAT signalling.

1.8 Examples of other immune receptors that utilise TCR-associated proteins

1.8.1. Prolactin receptor

Prolactin is a versatile hormone produced by the anterior pituitary gland, which binds its Prolactin receptor (PRL-R), which is part of the cytokine superfamily, which also includes the receptors for IL-2, IL-4 and IL-6. Prolactin receptors are distributed throughout the immune system are important in regulating proliferation as well as increasing the effect of IL-2 and stimulating production of IFNγ. PRL-R is associated with the JAK2/STAT5 pathway as well as other downstream pathways such as Ras/ERK.

The similarity in pathways in some of the pathways activated by Prolactin with pathways activated by the TCR prompted investigations into whether T-cell associated proteins were required for successful downstream signalling. Initially, Vav1 was been shown to be stimulated by prolactin in the Nb-2 rat T-cell line and maximally phosphorylated 5 minutes post-stimulation and it was also shown that Vav transiently translocated to the nucleus within 10-30minutes after stimulation (Clevenger et al., 1995). Following the

demonstration that Vav was phosphorylated in response to Prolactin, Zap70 was also then also shown to play a role at this receptor (Montgomery et al., 1998). In was shown to be phosphorylated with 1-2 minutes although the levels declined again by 5 minutes. It was also found that CD3 chains of the TCR associated complex were phosphorylated in response to prolactin when the chains were immunoprecipitated following prolactin stimulation and probed with an anti-phosphotyrosine antibody (Montgomery et al., 1998). These experiments clearly demonstrated cross-talk between the two receptors and demonstrated that TCR phosphorylation could occur when a distant receptor i.e. the prolactin receptor is stimulated.

1.8.2 CXCL12 Receptor

CXCL12 (or stroma cell-derived factor-1) is a chemokine that drives T-cells to areas where they are likely to encounter antigen through transendothelial migration. The receptor for this chemokine is a G-protein coupled receptor known as CXCR4. It has, like the IFNAR, also been shown to utilize a subset of T-cell receptor associated proteins such as CD45 (Fernandis et al. 2003), Zap70 (Ticchioni et al., 2002, Kumar et al., 2006), Lck (Ganju et al., 1998) and Slp76 (Kremer et al., 2003) and p52Shc (Patrussi et al., 2007).

Initial studies by Ticchioni et al., 2002, showed that CXCR4 stimulation with CXCL12 results in time-dependent phosphorylation of Zap70, Vav1 and ERK1/2. Since then experiments have demonstrated that Zap70 and Vav also form a complex with the p52Shc isoform and Lck upon SDF-1α treatment (Patrussi et al., 2007). Importantly, the absnce of any of the aforementioned proteins hampers the sustained ERK MAPK signalling normally brought about by this receptor (Tilton et al., 2000).

Ticchioni et al., 2002 also showed that absence of Zap70 impairs migration in P116, Zap70 deficient, Jurkat cells and the migration abilities can be restored to that of the same level as wild-type Jurkat cells upon reconstitution. Inhibiting Zap70 with the Syk kinase inhibitor piceatannol also impaired migrational responses. In addition, Zap70 absence or inhibition impaired phosphorylation of Vav and mutating Vav to a dominant negative form (L213A) decreased migration when transfected into Jurkat cells, demonstrating the role of Vav1.

Due to the array of shared signalling proteins commanded by the TCR and CXCR4 receptor, investigations were carried out to see if the TCR itself was required by the CXCR4 receptor. Indeed, it was established that the TCRβ chain is a pre-requisite for CXCR4 evoked ERK MAPK signalling as its absence halts the response. Furthermore, SDF-1α brings actually brings about phosphorylation of the TCRζ chain even though it is part of a separate receptor (Patrussi et al., 2007). This verifies that crosstalk takes place between the TCR and CXCR4 receptor.

1.9 AIMS OF PROJECT

Despite the IFNAR and TCR receptors eliciting opposing cellular responses when stimulated, it is now evident that share similarities in their mode of signalling in T cells as both receptors are able to bring about phosphorylation of the ERK1/2 MAP kinases. Additionally, in order for this pathway to ensue, the two receptors require an overlapping subset of proteins, as described in section 1.6.4. These proteins include CD45, Lck, Zap70 and Vav (Petricoin III et al., 1997, Ahmed et al., 2005, Platanias & Sweet, 1994, Uddin et al., 1997, Adam et al., 2000).

The aim of this thesis was to determine further similarities between the two receptors by looking at two key proteins in chapters 3 and 4 respectively, Slp76 and Vav, which are both essential components of the TCR early signalling complex. Since they are phosphorylated in a Zap70 dependent manner at the TCR (Wardenburg et al., 1996, Raab et al., 1997), which is a protein that is also required for signalling by the IFNAR, it seemed likely that may also be recruited to the IFNAR also. In the final results, chapter 5, the aim was to elaborate on the results of the previous results by deducing whether the TCR itself was essential for IFNAR-induced signalling, which would demonstrate that crosstalk occurs between the two receptors.

CHAPTER 2

Materials and Methods

2.1. Molecular Biology Techniques Used to manipulate DNA

All buffers in this section were prepared with distilled water from an Elga water purifier. In addition, buffers prepared for bacterial culture and DNA preparation were autoclaved before use or sterile filtered where indicated with a 0.2 μM filter (Millipore) in order to remove bacterial particles that are too large to pass through the filter. All chemicals used in this section were purchased from Sigma or VWR unless otherwise stated.

2.1.1 Bacterial Culture

2.1.1.1 Buffers used:

Luria-Bertani (LB) liquid growth medium

- 10 g/L NaCl
- 10 g/L Tryptone
- 5 g/L yeast extract

LB Agar solid growth medium

- 10 g/L NaCl
- 10 g/L Tryptone
- 5 g/L Yeast Extract
- 20 g/L agar

NZY+ broth (amounts per Litre)

- 10 g NZ amine (casein hydrolysate)
- 5 g yeast extract
- 5 g NaCl
- 12.5 ml 1M $MgCl_2$
- 12.5 ml 1M $MgSO_4$
- 20 ml of 20% (w/v) glucose

Antibiotics used (all sterile filtered)

Carbenicillin – 250 mg/ml dissolved in water

Kanamycin – 50 mg/ml dissolved in water

Chloramphenicol – 34 mg/ml dissolved in 70% ethanol

Tetracyclin – 5 mg/ml dissolved in 70% ethanol

2.1.1.2 Bacterial strain used

In all of section 2.0 the XL-1-blue *E.Coli* bacterial strain was used (originally produced by Stratagene), which is a strain widely used for propagation and cloning. The genotype is listed as *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17*(rK-, mK+), supE*44, *relA*1, *lac,* [F', *proAB, lacIqZ*ΔM15::Tn10(tetr)]. This strain is both nalidixic acid resistant and tetracycline resistant (carried on the F plasmid).

2.1.1.3 Culturing bacteria

Growing bacteria in liquid media: Starter cultures

For all minipreps and for growing up cloned bacterial colonies, a single transformed colony was picked and added to 5 mls of sterile LB media to which 5 µl of the appropriate antibiotic in stock concentrations listed in section 2.1.1.2 was added to make a 1:1000 dilution. Cultures were then sealed and grown in 225rpm rotation overnight

Growing bacterial colonies on solid media

Following bacterial transformation, bacteria were plated onto sterile 10 cm diameter Petri dishes containing 25 mls dry agar plus antibiotic, which had been pre-poured whilst warm and allowed to set. Plates were then incubated overnight at 37ºC.

2.1.2 Preparation of glycerol stocks

A 700 µl aliquot of a 5ml starter culture was transferred to a cryovial. 300 µl of sterile 70% glycerol solution was then added. Cells were stored at -80ºC.

2.1.3 Preparation of competent bacterial cells

DNA is a very hydrophilic molecule so it normally can not pass through a bacterial cell's membrane. In order to allow plasmid DNA entry into bacteria, they must first be made "competent" to take up DNA. This is done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium, in the presence of rubidium chloride.

2.1.3.1 Buffers used for preparing competent cells

TFB1 Buffer

- 100mM rubidium chloride
- 50mM manganese chloride
- 20mM potassium acetate
- 50mM calcium chloride
- 15% (w/v) glycerol
- adjusted to pH 5.8. with acetic acid

TFB2 Buffer

- 10 mM MOPS
- 10 mM rubidium chloride
- 75 mM calcium chloride
- 15% (w/v) glycerol
- adjusted to pH. 6.8 with potassium hydroxide

2.1.3.2 Protocol for preparing competent cells

XL-1-blue bacterial cells were streaked on a tetracycline-containing agar plate from a glycerol stock and incubated overnight at 37ºC. A single colony was then picked and grown over night in tetracycline-containing LB. The following day, 1ml of this culture was inoculated into 100 ml LB containing tetracycline, and grown to an optical density of 0.42-0.45 (measured at 595 nm using a 1 ml plastic cuvette in a spectrophotometer). To stop the cells from further growth, the flask was cooled on ice for 5 minutes. The cells were then pelleted by centrifugation at 4000 rpm for 5 minutes. The supernatant was removed and 30 ml of ice cold TFB1 buffer was added to resuspend the cells. This TFB1/bacterial suspension was left on ice for 90 minutes. The cells were then centrifuged for a second time at 4000 rpm for 5 minutes and the supernatant was eradicated. Bacterial cells were resuspended in 4ml ice cold TFB2 buffer and pipetted into 100µl aliquots to be stored at -80ºc.

2.1.4 Transformation of bacterial cells to take up plasmid DNA

A 100 µl aliquot of competent XL-1-blue cells (refer to section 2.1.3 for preparation) was thawed on ice. Once thawed half of the aliquot i.e. 50 µl XL-1-blue cells was split into a thin-walled round bottom polypropylene tube. 50-100 ng DNA was then added to 50 µl cells.

The following incubation periods then followed; 20 minutes on ice, 45 seconds at 42°C to heat pulse bacteria so that they would take up plasmid DNA, and finally 2 minutes on ice. 900 µl of sterile LB broth was then added to each tube and the cells were placed in 37°C incubator for 60 minutes, rotating at a speed of 225 rotations per minute (rpm) in order to

allow the cells to recover. The cells were then spun down at 13 000 rpm for 2 minutes and 900 µl of the supernatant was removed. The cells were resuspended in the remaining 100µl and plated onto an agar plate containing the appropriate antibiotic (see section 2.1.1.4 for preparation of agar plates). Agar plates were then incubated overnight in a humidified 37°C incubator.

2.1.5 Plasmid vectors used

(a) pcDNAhygro3.1 (+/-) (Invitrogen). This is a 5597 nucleotide Mammalian expression vector with a CMV promoter (bases 209-863), T7 promoter/priming site (bases 863-882), multiple cloning site (bases 895-1010), BGH reverse priming site, SV40 promoter (bases 1776-2100), f1 origin (bases 1298-1711), hygromycin resistance gene (2118-3141), pUC origin (bases 3786-4456) and an ampicillin reistance gene (bases 4601-5461)

(b) pcDNA3.1-mRFP-C fusion vector (see Ahmed et al. 2008 for description). This is a 6110 nucleotide vector, created using the pcDNA3.1(+) described above (with neomycin resistance rather than hygromycin resistance). A monomeric RFP gene was been cloned in frame at the C-terminus of the multiple cloning site between the NotI and XhoI restriction sites.

(c) pcDNA3.1-mRFP-N fusion vector – As described above only the mRFP was cloned into the vector at the N-terminal of the multiple cloning site between the NheI and HindIII sites.

(d) eGFP-C1 (BD Biosciences Clontech) – This vector encodes a red-shifted variant of wild-type GFP, which has been optimised for brighter fluorescence and higher Mammalian expression.The eGFP is this plasmid lies in reading frame 2, N-terminal to the multiple

cloning site. Features of this plasmid include a CMV immediate early promoter, a multiple cloning site, a SV40 polyadenylation signal, f1 DNA origin, Kanamycin resistnace gene, SV40 origin of replication and early promoter, a neomycin resistance gene, a HSV polyadenylation signal and pUC plasmid replication origin.

(e) eGFP-N3 (BD Biosciences Clontech) – As in (d) but the eGFP gene lies C-terminal to the multiple cloning site in the third reading frame.

2.1.6 Constructs used in this thesis

(a) eGFP-Vav-c1 - This construct was obtained from the laboratory of A.Ridley. Vav is cloned between the BglII and EcoRI sites in frame. This vector is described in Kranewitter & Gimona, (1999).

(b) eGFP-Vav-hygro3.1 - Using this construct, the eGFP-Vav cassette was cloned into pcDNA3.1/hygro. Firstly Vav was cloned in frame into pcDNA3.1(-)/hygro between the XhoI and BamHI sites. Secondly an eGFP-C3 tag was amplified using PCR. The tag was then cloned into the eGFP-Vav3.1/hygro vector using the XhoI enzyme only.

The eGFP tag primer used were (in the 5' to 3' direction):

Forwards: GAA CCG TCA GAT CCG CTC GAG CTA CCG GTC (T_m = 73.6ºC)

Reverse: CAG TTA TCT AGA TCC GGT GGA TCC CGG (T_m= 68.0ºC)

(c) Slp76-hygro3.1-RFP-C: Slp76 DNA obtained originally from the laboratory of L.E. Samelson and was cloned in frame into the pcDNA3.1/hygro-RFP-C vector between the NheI and XhoI restriction sites.

(d) IFNAR-eGFP-N3 - IFNAR was originally obtained from the laboratory of Giles Uzé, cloned into the eGFP-N3 vector with EcoRI and BglII. The IFNAR gene was amplified

through PCR using the following primers, which read in the 5'3' direction. Underlined is a BglII site in the forward primer and EcoRI site in the reverse primer:

Forwards: GAA GAT CTT ATG GTC GTG CTC CTG GGC GCG ACG

Reverse: CGG AAT TCC GTA CAA AGT CCT GCT GTA GTT CTT C

(e) Zap70-hygro3.1-RFP-N – Zap70 was cloned from a Zap70-pcDNA3.1-RFP-N vector (see Ahmed et al., 2005 for description of original Zap70-containing plasmid). The entire Zap70-RFP-N cassette was cloned in frame into the pcDNA3.1/hygro (+) vector between the NotI and the NheI restriction sites so that the vector would be hygromycin rather then neomycin resistant.

2.1.7 Primers used for sequencing constructs

(a) BGH reverse primer: Used to reverse sequence inserts in the MCS of pcDNA3.1.

TAG AAG GCA CAG TCG AGG

(b) T7 promoter forward primer: Used to check for inserted DNA in the MCS of pcDNAhygro3.1 by sequencing from the N-termal T7 promoter. Melting temperature = 64.2ºC

TAA TAC GAC TCA CTA TAG GG

(c) GFP/RFP reverse primer: Used for sequencing the junction between the C-terminus of DNA of a DNA fragment inserted into C-terminal RFP/GFP tag vector

GCC CTC GCC CTC GCC

(d) GFP C-terminal forward 3 primer: Used for sequencing the junction between the DNA insert and an N-terminal GFP tag. Melting temperature = 55.4ºC.

CAT GGT CCT GCT GGA GTT CG

(e) eGFP-N3 forward primer: Used to sequence junction between multiple cloning site and IFNAR1 sequence

G TGA ACC GTC AGA TCC GCT AGC

(f) IFNAR1 Reverse primer: Used to sequence the C-terminus of and the C-terminal eGFP-N3 tag. Melting temperature = 55ºC

CAT AAT TGA TGC ATC TCA AGA AG

2.1.8. Amplification of genes using a Polymerase chain reaction (PCR)

This is a method to amplify a gene of interest from a small amount of plasmid DNA containing the desired gene. For all reactions Vent® polymerase was used (NEB), which is a high fidelity (error rate of 57 x 10^{-6} bases) thermophilic DNA polymerase purified from *E.coli* with integral proofreading exonuclease activity in the 5' to 3' direction. The following reactions were set up:

50µl PCR reaction mix

5 µl (20-25 µg) template DNA

5 µl 10 µM forward primer

5 µl 10 µM reverse primer

1 µl 10 mM dNTPs (2.5 mM dATP, 2.5 mM dCTP, 2.5 mM dGTP, 2.5 mM dTTP)

5 µl Vent polymerase buffer (NEB)

32 µl ddH_2O

1 µl Vent® polymerase (NEB)

Thermal Cycles for PCR

One initial melting DNA cycle: 95ºc - 5 minutes

Melting: 95ºc – 30 seconds
Annealing: calculated from annealing
temperatures of primers – 30 seconds
Extension: 72ºc – 3.5 minutes
} x 30 cycles

The PCR product was then electrophoresed on a 0.8% agarose gel to check that it was the correct size. It was then excised and gel purified to eliminate unwanted parental plasmid DNA template and dNTPs that would remain in the reaction mix (see sections 2.1.9.4 and 2.1.9.5 for gel purification and gel electrophoresis protocols).

2.1.9 Cloning of a gene of interest into a DNA plasmid construct

2.1.9.1 Preparation of DNA plasmids

A 5 ml starter culture (refer to section 2.1.1.3.1) of bacteria containing the plasmid to be purified was centrifuged for 5 minutes at 4000 rpm to pellet the bacterial cells. The supernatant LB was removed and the pellet was then used for DNA extraction using the QIAquick spin miniprep kit (Qiagen). The protocol was carried out according to the manufacturers instructions using the equipment and buffers provided. A 5 ml starter culture yields 20-50 µg plasmid DNA depending on copy number of the plasmid. The principal behind the miniprep kit is that firstly bacterial cells are lysed under alkaline conditions, followed by adsorption of DNA onto silica in the presence of high salt. The bound DNA

from the cleared lysate is then washed and eluted from the silica membrane to give purified DNA in solution.

2.1.9.2 Restriction Digests

Restriction digests were set up and carried out according to the manufacturers' instructions (New England Biolabs). For each restriction enzyme or enzyme combination, a buffer was recommended by the manufacturer in order to aid digestion. All restriction enzymes were purchased from New England Biolabs. Standard dilutions of buffers, DNA and enzymes were also carried out according to the manufacturers' guidelines. In accordance with this, the following reactions were set up:

Restriction Digest Reactions (total 20µl volume)

14 µl of vector or PCR amplified DNA

2 µl 10x buffer (so that a 1x final reaction volume was achieved)

2 µl 10x bovine serum albumin (dilute from a 100X BSA stock from NEB)

1 µl of restriction enzyme (to give a final volume of 10000-20000 U/ml)

The reagents above were mixed and incubated in a 37°C water bath for 2 hours for the reaction to proceed.

2.1.9.3 Dephosphorylation of 5' ends of digested vector DNA

In some cases, only single enzyme was required for a restriction digest rather than two. In order to prevent single digested plasmids from religating before insertion of the digested DNA fragment can occur, 1 µl (10 000 U/ml) of calf intestinal phosphatase (NEB) was

added to each 20 µl digest to remove 5' phosphates. Each digest was then subjected to a further incubation period of one hour at 37ºC.

2.1.9.4 Using agarose gel electrophoresis to separate digested DNA fragments

6x loading dye	0.5x TBE buffer pH.8.3
- 60% glycerol solution	- 1 mM EDTA
- 0.05% bromophenol blue	- 45 mM trizma borate

In order to isolate the target gene fragment, DNA was subjected to agarose gel electrophoresis. This separates DNA according to size in an electric field, which is applied across the agarose gel. Polymerised agarose forms a pore-containing matrix through which DNA of smaller mass can migrate more easily than heavier fragments meaning that fragments are separated by size with the smaller ones travelling further. All fragments were separated on a 0.8% horizontal agarose gel in 0.5% TBE, to which ethidium bromide was also added. To the 20 µl restriction digest, 5 µl of 6x loading dye was added and mixed. The digests were then loaded into the agarose gel wells along with a 1kB molecular size marker (NEB). The gel was then covered with 0.5x TBE buffer and run at 80V for one hour. The ethidium bromide bound DNA was visualised using a UV transilluminator. Bands of correct size were excised.

2.1.9.5 Gel extraction of digested DNA from an agarose gel

In order to purify the DNA from excised agarose gel fragments and removes contaminants, a QIAquick gel extraction kit (Qiagen) was used according to the manufacturers instructions, using the buffers provided. These binding buffers provide the correct salt and pH for the DNA to bind to a silica membrane in a column. The purified DNA is then eluted from the column using the elution buffer provided and can be used for further manipulation.

2.1.9.6 Ligation of plasmid vector and insert

The concentration of vector and insert DNA was quantified through use of a nanodrop ND-1000 spectrometer (Labtech) connected the appropriate software that gives the DNA concentration in 1 µl well mixed DNA sample. The number of µl of vector and µl insert was calculated so that 100 ng vector was used (for plasmids around 5 kb). The appropriate quantity of insert DNA was then added to make up the volume to 17 µl. If using this ratio was unsuccessful, the ratio was adjusted so that either more insert or less insert was used.

Ligation reactions set up (total 20µl volume)

Number of µl digested, gel purified insert DNA to make volume up to 17 µl

100 ng vector backbone

2 µl 10x ligase buffer (defroseted on ice)

1 µl T4 DNA ligase (400 000 U/ml) purchased from NEB

2.1.9.7 Transformation of bacterial cells to take up cloned DNA

10 µl of ligated DNA or half of the total volume was added to 50 µl of thawed competent XL-1-Blue bacterial cells. A standard bacterial transformation protocol was then followed (see section 2.1.4) in order to induce the cells to take up ligated plasmid DNA.

2.1.9.8 Isolating bacterial colonies with cloned construct plasmid

Rapid screening lysis buffer (dissolved in dH_2O)

- 5 mM EDTA
- 10% (w/v) sucrose
- 0.35% (w/v) SDS
- 100 mM NaOH
- 60 mM KCl
- 0.05% bromophenol blue

A "rapid screening" protocol was used to quickly assess, from a large number of colonies, which ones are likely to contain a plasmid with insert and which ones contain only religated plasmid. This involves lysis of bacteria so that all DNA and RNA can be electrophoresed on a 0.8% agarose gel and different sized components can be assessed.

Firstly colonies of bacteria were picked and expanded in size by streaking them in small, numbered spots on a fresh antibiotic-containing agar plate, which was incubated at 37ºC overnight. The following day part of each streak was resuspended in 30 µl rapid screen buffer and vigorously pipetted up and down. Each lysed colony was then placed on ice for 5 minutes at 37ºC. Debris was then pelleted through centrifugation at 13 000 rpm. 15 µl of

the supernatant was then loaded and electrophoresed on a 0.8% agarose gel. 1µl of original vector in 5 µl loading dye was also loaded as a control. The size of the DNA bands for each bacterial colony was compared to the original plasmid and clones were chosen that had lower mobility bands since this is likely due to the fact that they harboured a plasmid with insert and therefore the DNA migrated slower due a greater size.

2.1.9.10 Checking that plasmids have insert

The overnight cultures of selected colonies were centrifuged at 4000 rpm to pellet cells and then the QIAprep spin miniprep kit was used to purify the plasmid DNA (see section 2.1.10.1). Restriction digests were then set up using the same enzyme(s) as used for the original digestion (see section 2.1.10.2). Digested DNA was then subjected to gel electropheresis (see section 2.1.10.4) and visualised using a UV transilluminator. If an insert was visualised of correct size, the plasmid was sent for sequencing using the appropriate forward or reverse primers to check that the sequence of the insert was correct at the junction with the plasmid and that it was in the correct frame. Also fusion proteins e.g. GFP or RFP were checked for through sequencing (see section 2.1.8 for list of sequencing primers).

2.1.9.11 Sequencing DNA

DNA was sequenced at a specialised facility at Wolfson Institute for Biomedical Research, which is part of University College London. Miniprep plasmid DNA was diluted to a concentration of 150 fmol and primers were diluted to a concentration of 12 fmol, in accordance with the specifications of the institute

2.1.10 Site-directed mutagenesis

2.1.10.1 Primers used for mutagenesis

Name	Mutation	Primer Sequence
Slp76 Y112F Forward	A to T base substitution	GGT CGT CCT TTG AAG AAG ACG ATT TTG AAA GTC CCA ATG ATG ACC
Slp76 Y112F Reverse	T to A base substitution	GGT CAT TGG GAC TTT CAA AAT CGT CTT CTT CAA AGG ACG ACC
Slp76 Y128F Forward	A to T base substitution	GAG GAT GAT GGA GAC TTT GAG TCC CCC AAT GAG
Slp76 Y128F Reverse	T to A base substitution	CTC ATT GGG GGA CTC AAA GTC TCC ATC ATC CTC
Slp76 Y145F Forward	A to T base substitution	GAA GAT GAC GCG GAT TTT GAG CCG CCA CCC TCC ATT G
Slp76 Y145F Reverse	T to A base substitution	CTA AGG AGG GTG GCG GCT CAA AAT CCG CGT CAT CTT C
Vav Y174F Forward	A to T base substitution	GAA GGC GAC GAG ATC TTT GAG GAC CTC ATG CGC
Vav Y174F Reverse	T to A base substitution	GCG CAT GAG GTC CTC AAA GAT CTC GTC GCC TTC
Vav SH2 domain R696K Forward	CG to AA substitution	GGG ACT TTC TTG GTG AAG CAG AGG GTG AAG GAT GC
Vav SH2 R696K domain Reverse	CG to AA substitution	GCA TCC TTC ACC CTC TGC TTC ACC AAG AAA GTG CC
Zap70 Y315F Forward	A to T base substitution	GAC ACG AGC GTG TTT GAG AGC CCC TAC
Zap70 Y315F Reverse	T to A base substitution	GTA GGG GCT CTC AAA CAC GCT CGT GTC

2.1.10.2 Protocol for mutagenesis

Site directed mutagenesis was used to mutate nucleic acid residues within a codon of an amino acid so that the amino acid expression was changed. Mutations were made at critical tyrosine residues in Slp76-RFP, Zap70-RFP and eGFP-Vav constructs so that an amino acid change from a tyrosine to a phenylalanine took place. Also a double base mutation was carried out within the C-terminal of eGFP-Vav so that an arginine amino acid was substituted for lysine within the SH2 domain.

In order to carry out this mutategenesis the "Quikchange" mutagenesis kit was utilised (Stratagene) and all steps were carried out in accordance with the manufacturer's instructions. These instructions included specifications of primer design, which were required to have a melting temperature of about 78°C and be 25-45 amino acids in length (see section 2.2.1 for list of all primers). For each reaction *PfuTurbo* DNA polymerase (Stratagene), which is a high fidelity enzyme that replicates both strands of the template without displacing the mutant oligonucleotide primers. This polymerase transcribes PCR reactions of 50 µl volume were set up as described below according to the protocol and then were subjected to the thermal cycles indicated.

Reaction Mix:

5 µl 1 µM forward primer and 5 µl 1 µM reverse primer

2 µl 10 mM dNTP mix (NEB) containing 2.5 mM of dATP, dCTP, dGTP and dTTP

1 µl template DNA (approximately 10 ng/µl)

5 µl 10x Pfu Turbo buffer

31 µl ddH_2O

1 µl Pfu DNA polymerase (Stratagene), which was added last to the mix

Thermal Cycles:

Initial melting cycle: 95ºC for 5 minute

Melting: 95ºC for 30 seconds
Annealing: 55ºC for 1 minute
Extension: 68ºC for 20 minutes
} x 20 cycles

Following PCR, a mixture of both template non-mutated parental DNA and newly synthesised mutated plasmid DNA remains and so it is necessary to eliminate the unwanted non-mutated template. This is achieved through use of the Dpn1 endonuclease enzyme (NEB), which recognises and digests methylated parental double stranded DNA. Since this originates from bacteria, it will have undergone methylation modifications within the cells whereas the newly synthesised DNA will be in unmethylated, "nicked" form. To this end, 1µl of Dpn1 (20000 U/ml) was added to each reaction, followed by an hour long incubation period at 37ºC. 10 µl of each reaction volume was then run on a 0.8% agarose gel to check the presence the reactions were successful and that a correctly sized plasmid product could be visualised under a UV transillumninator. 10 µl of the reaction was then used to transform 50 µl of competent XL-1-blue cells according to the protocol in section 2.1.4. However, instead of using LB for the hour incubation recovery period, NZY+ broth was used instead (see section 2.1.1.1 for composition) as recommended by the manufacturer.

2.1.11 Preparation of DNA for transfection of Mammalian cells using the Midiprep protocol

Although DNA can be prepared in small quantities using the Quaigen miniprep protocol, this does not generate enough DNA for Mammalian cell transfection (see section 3.4) and so the midiprep protocol was used for preparing a larger quantity of concentrated DNA.

2.1.11.1 Buffers used for midiprep

Buffer P1

- 25 mM Trizma hydrochloride
- 10 mM EDTA
- Dissolved in dH_2O and adjusted to pH 8.0 with KCl

BufferP2

- 200 mM NaOH
- 1% SDS

Buffer P3

- 3 M Potassium actetate in dH_2O
- Adjusted to pH 5.5 with glacial acetic acid

TE Buffer (pH.8.0)

- 10 mM Trizma base
- 1 mM EDTA

2.1.11.2 Protocol for midiprep preparation of DNA

A 5 ml starter culture of bacteria containing the plasmid DNA to be purified was grown overnight in LB with the appropriate antibiotic added. 1 ml of the starter culture was then added to 100 ml sterile LB liquid growth with the appropriate antibiotic added, and grown overnight in a 37°C incubator rotating at 225 rpm. The culture was then spun for 20 minute at 4000 rpm.The supernatant was then removed so that only the pelleted bacterial cells remained

To the bacterial pellet, 5 ml of buffer P1 was used to resuspend the cells. 5 ml of buffer P2 was then added and gently mixed to lyse the cells. Finally 5 ml buffer P3 was added and gently mixed, which results in precipitation of the insoluble cell fragments. The cells were then spun for 30 minutes at 4000 rpm in order to pellet the insoluble precipitate.

The soluble supernatant was removed and decanted to a fresh tube and an equal volume of phenolchloroform isoamid alcohol and vortexed for approximately 1 minute until the mixture appeared "milky" in colour. The mixture was then centrifuged at 4000 rpm to separate the two immiscible layers. The top layers was then removed and transferred to a fresh tube, to which 2 volumes (30 mls) of 100% analytical grade ethanol was added to precipitate the DNA. This was mixed through inverting 10 times. Centrifugation at 4000 rpm then followed for 30 minutes in order to pellet the DNA. The supernatant was carefully removed and the pellet was washed through the addition of 70% analytic grade ethanol. This was then removed and the pellet was left to air dry at room temperature. Once dried the pellet was suspended in 500 µl TE buffer containing 10 µg/ml RNAase (Quaigen) to digest all RNA. This was incubated for one hour at 37ºC.

The next procedure involved the polyethylene glycol (PEG) precipitation of DNA. 125 µl of 4 M sterile NaCl was added to the 500 µl resuspended DNA and vortexed thoroughly. 1ml of sterile 13% PEG 8000 solution was then added and gently mixed through inversion. The mix was then left to precipitate for 2 hours on ice and centrifuged at 13000 rpm for 20 minutes. The supernatant was discarded and two washing steps followed. The first was with 1ml 70% ethanol solution, which was then removed and replaced with the second wash of 100% analytical grade ethanol. This was removed and the remaining DNA pellet

was left to air dry. Once dried the pellet was resuspended in 200-300 µl of autoclaved distilled water and stored at -20°C until use.

2.1.11.3 Measurement of DNA concentration

DNA concentration was determined by adding 5 µl of the DNA to 495 µl ddH_2O to give a 500 µl volume in a glass walled quartz cuvette. The absorbance of the sample was the determined using a Cary 100 bio spectrophotometer (Varian) set at a wavelength of 260 nm. The concentration was calculated using the following formula (based on the fact an OD_{260}=1 is equivalent to a DNA concentratiom of 50 µg/ml). The total was divided by 1000 to give the concentration in µg/µl:

Concentration = absorbance at 260 nm x 50 x dilution factor

(5 µl DNA was used in a 500 µl cuvette, therefore dilution factor is 100)

2.2 Techniques used to culture and manipulate transformed cell lines

2.2.1 Use of Jurkat cells and Jurkat cell derivatives to study signalling

Jurkat cells have been extensively used to study T-cell signalling and through their use, many important discoveries have been made. They were first isolated in the 1980s where screening T cell lymphoma cell lines since they are able to spontaneously produce IL-2 and, because of this, they are easy to maintain in culture as they continue to replicate in a similar manner to that which occurs during clonal expansion during an immune response and do not require addition of extra growth factors. Since then, Jurkat cell derivatives have

been created that lack expression of a particular protein. These were initially created through the exposure of Jurkat cells to a mutagen such as ethylmethanesulphonate or the frameshift mutagen ICR-191. Cells were then screened based on signalling properties such as CD69 marker expression, TCR-dependent calcium responses and protein tyrosine phosphorylation patterns. Individual clones were isolated that lacked expression of particular cell lines (reviewed in Abraham & Weiss, 2004). Other cell lines have also been created that lack expression of a particular protein through disruption of alleles using of a plasmid targetting cassette. Although there are limitations to their use e.g. the lack of the phosphatase PTEN, they are advantageous due the ease of maintenance and also creating knockout or mutant mouse models is much more time consuming and costly. Additionally some knockout mice develop T-cell developmental blocks due to the absence of key signalling proteins and therefore no peripheral T cells exist to be studied. Therefore the way to circumvent this problem is through use of knockout lymphoma cell lines.

2.2.2 Cell Lines Used

Name	Source	Phenotype and reference
Jurkat E6.1	ECACC	Isolated from a human donor in 1980 with T cell lymphoma. Clone E6-1 cells produce large amounts of IL-2 after stimulation. (Gillis & Watson, 1980)
JRT3-T3.5	ATCC	TCRβ deficient (Weiss & Stobo, 1985)
PF2.4	A.Weiss	TCRβ deficient cells reconstituted with a TCRβ encoding expression vector (Ohashi et al., 1985)
J.Vav1	R. Abraham	Vav deficient through use of a Vav G418 resistant Vav gene targeting vector (Cao et al., 2002)

Name	Source	Phenotype and reference
J.Vav1 reconstituted (clone 15-11)	R. Abraham	Vav deficient cells reconstituted with a Vav expression vector (Cao et al., 2002)
J14	A.Weiss	Slp76 deficient (Yablonski et al., 1998), isolated while screening mutagenised Jurkat T cell subclones
J14 reconstituted with Slp76-RFP	A.Weiss, transfected by myself	Slp76 deficient transfected stably with an Slp76-RFP expression vector
J14 reconstituted with 3YF Slp76-RFP	A.Weiss, transfected by myself	Slp76 deficient transfected stably with an Y3F mutant Slp76-RFP expression vector
P116	A.Weiss	Zap70 deficient (Williams et al., 1998)

2.2.3 Maintaining cells in culture

All cell lines detailed in section 3.2 are suspension cells that grow in clumps and do not adhere to surfaces. They were therefore cultured in different sized specialised filter capped sterile plastic flasks (TTP or Sarstedt) depending on the numbers of cells required for experimentation. All cell culture was carried out using aseptic techniques in a filter flow shielded cabinet (HeraSafe Heraeus). The media used for maintaining and growing the cells was RPMI-1640 containing L-glutamine (Cambrex). This media was additionally supplemented with 10% heat-inactivated, gamma-radiated fetal bovine serum (Biosera) and antimycotic/antibiotic (BioWhittaker). From herein this is referred to as "supplemented RPMI". Cells were maintained in a humidified incubator with 5% (v/v) carbon dioxide at 37°C at a density of 5 x 10^5 to 1 x 10^6 per ml. The J.Vav1 cells and reconstituted J.Vav1 cells have been stably transfected with DNA constructs with a G418 resistance gene and so

to maintain selection were cultured in the above media, supplemented with 500 μg/ml G418 (diluted from a 100 mg/ml liquid stock from Autogen Bioclear). J14 cells reconstituted with Slp76-RFP or 3YF Slp76-RFP were cultured in 100 μg/ml hygromycin (diluted from a 100 mg/ml liquid stock from Autogen Bioclear) to maintain selection for these plasmids since both plasmids encompassed a hygromycin resistance gene. Every 2-3 days the cells were split into a fresh flash with fresh supplemented RPMI media.

2.2.4 Transfection of cell lines using electroporation

In order to induce cultured cells to take up plasmid DNA so that they would express a protein of interest e.g. a fluorescently tagged protein or mutated protein, I used the electroporation protocol. This basis of this method is that applying a short burst of externally applied electric current increases the permeability of the cell membrane, causing pores to form through disruption of phospholipids within the plasma membrane. This allows polar DNA plasmids to enter the cells and hopefully and incorporate into the cell genome. The cells are then allowed to recover in complete growth medium and the pores quickly reseal.

Initially cells were grown in culture to a density of approximately 5 x 10^5 cells/ml. In order to check the precise number of cells, a Neubauer double cell hemocytometer (Calbiochem) was used to count the cells under a microscope according to the manufacturers' instructions. The number of millilitres of cells required for 10 to 20 million cells was calculated. This volume was then centrifuged at 1000 rpm for 5 minutes. The supernatant was then removed and replaced with an equal volume of RPMI only (without any supplements added) to wash the cells. The cells were then centrifuged again for 5 minutes

at 1000 rpm. The supernatant was removed and the cells were resuspended in 350 µl unsupplemented RPMI. The cells were then transferred to a 4mm electroporation chamber cuvette (Eurogenetic) and 50 µg-100µg of transfection grade plasmid DNA was then added (prepared using the midiprep protocol in section 2.1.11) and mixed gently. The chamber containing the cells plus DNA was then pulsed (Biorad gene pulser) set at a charging pulse of 0.27 V and a capacitance of 960 µF. Cells were then left to equilibriate and recover for 10 minutes at room temperature. The transfected cells were then resuspended in 20 ml supplemented RPMI media. After 24 hours the media was replaced and, if the cells were being used to gain a stable population of transfected cells, the appropriate antibiotic was added to select for cells encoded the plasmid-derived resistance gene.

2.2.5 Selecting cells that are expressing plasmid DNA to gain stably expressing cell lines

Cells were transfected with Mammalian expression vectors through electroporation as described in section 2.2.4. Each plasmid vector carries an antibiotic resistance gene hence when the appropriate antibiotic is added only cells containing transfected plasmid DNA survive. Cells were selected initially in 2 mg/ml G418 or 400 µg/ml hygromycin for 2-3 weeks. These amounts had been pre-selected through use of a "kill curve". The minimum antibiotic concentration for 100% cell death of untransfected cells was selected to be used for future selection of cells. Following 2-3 weeks culture of a transfected cell population in the presence of antibiotic, expression of the desired protein was checked on a western blot (see section 2.2.10). If the protein was correctly expressed, a stable pools of cells could then be cloned out on 96 well plates through dilution cloning in order that single cell

clumps could be selected and cultured so that protein expression was equal in all cells in this culture. Clones were selected which had the desired expression level of protein. Once a cell population was stably expressing the desired plasmid, the antibiotic concentration added to the media could be halved.

2.2.6 Freezing cells and recovering frozen cells

A 20ml culture of 50-60% confluent cells were transferred from the culture flask to a 50 ml sterile falcom tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was aspirated and 20 ml pH7.4 1x PBS was added to wash the cells. The cells were centrifuged again at 1000 rpm and the supernatant was aspirated. 5 ml of supplemented RPMI was then used to resuspend the cells. Additionally 5 ml of freezing media, composed of 20% cell biology grade DMSO (dimethylsulphuroxide) with 80% FBS, was added and gently mixed. 1 ml aliquots were then transferred to capped cryovials and transferred to a -80°C freezer overnight. The following day, the cells were transferred to permanent liquid nitrogen storage (approximately -174°C).

When frozen cells were required for culturing, the appropriate cryovial was removed from liquid nitrogen storage and defrosted in a 37ºC water bath. The cells within the vial were then transferred to 20 mls of supplemented RPMI media that had been pre-warmed to 37ºC. The media was then replaced with fresh supplemented RPMI after 1-2 days to remove DMSO and cells were maintained as in section 2.2.3.

2.2.7 Stimulation of Cell lines through the TCR or through the IFNAR

Lysis Buffer (stored at 4ºC)

- 50 mM HEPES pH 7.5
- 1% NP40
- 1 mg/ml Bacitricin
- 10 mM NaF
- 10 mM $NaVO_4$
- 10% (w/v) Glycerol
- 50 mM NaCl
- 1 mM EDTA

Immediately prior to use 1% (v/v) protease inhibitor cocktail III (Calbiochem) was added and 2% 100mM PMSF. These steps were in order to stop protease degradation of proteins of interest.

For normal western blot analysis of proteins and immunprecipitations, cells were grown to about 50-60% confluency. Before being used, suspension cells were pelleted at 1000 rpm for 5 minutes and the supernatant was removed. The cells were then washed in an equal volume of 1 x Dulbecco's PBS (Biowhittaker) and centrifuged again for 5 minutes at 1000rpm. The cells were then resuspended in starvation media (RPMI containing L-glutamine (Cambrex), supplemented with antimycotic/antibiotic, but without any FBS added). 3-5 x 10^6 cells were then used per time point for Western blot analysis and cells were plated onto 6 cm diameter round plastic dishes (Sarstedt) since the cells adhere to the

bottom of the dish during the starvation period. For immunoprecipitation 10 x 10^6 cells were used per time point and cells were plated onto 10 cm diameter plastic dishes (Starsedt). The cells were then incubated at 37ºC for 2-4 hours. Cells were then stimulated with either 1 mg/ml OKT3 or 6000 units/ml of Roferon (Roche), which is a commercially available IFNα-2 protein, and incubated at 37°C for the time periods required. Cells were then lysed in NP-40 ice cold containing lysis buffer. All lysis was carried out on ice to avoid degradation of proteins. For cells on 6 cm dishes 400 µl of lysis buffer was used and for cells on 10cm dishes, 1ml lysis buffer was used. The collected cell suspensions were then centrifuged at 13 000 rpm for 20 minutes to pellet insoluble cell debris. The supernatant was then removed and placed in a fresh tube. Lysates were stored at -80ºC.

2.2.8 Use of the Bradford Assay technique to determine protein concentration

In order to determine the protein concentration of whole cell lysates, the Bradford assay reagent (Bio-Rad) was implemented according to the manufacturer's instructions. The Bradford dye assay is based on the equilibrium between three forms of Coomassie Blue G dye. Under strongly acid conditions, the dye is most stable as a doubly-protonated red form. Upon binding to protein, however, it is most stable as an unprotonated, blue form. Coomassie blue dye binds to primarily to basic and aromatic residues, especially arginine. Protein binding leads to a shift in the absorbance maximum from 465 nm to 595 nm.

1ml cuvettes were used for all measurements. To each, 800 µl dH_2O was added along with 200 µl of the Bradford Assay reagent. Lastly 5 µl of a protein containing sample e.g. whole cell lysate, was added and the cuvette was vortexed. The cuvettes were left for 5 minutes before measurement in order to allow time for the dye to bind to the protein.

Before I could determine the concentration of my protein samples, I constructed a standard curve using increasing defined concentrations of a bovine serum albumin (BSA) stock (NEB) between 0 and 10mg/ml. The optical density was measured at 595 nm using a Cary 100 spectrophotometer (Varian) and the readings gained were plotted graphically against protein concentration. The equation of the line of best fit could then be used to determine unknown protein concentrations in future based on the OD. The equation from my graph, which I used in all future experiments, was:

$$y = \frac{(x - 0.042)}{0.037}$$

y = protein concentration

$x = OD_{595nm}$

2.2.9 Separation of proteins in whole cell lysates through SDS-PAGE

2.2.9.1 Buffers used for SDS-PAGE

7.5% Resolving Gel Premix (7.5 % RGP)

- 37.5 ml 1.5 M Tris pH 8.8 stock solution
- 1.5 ml 10% SDS
- 73 ml distilled water

7.5% Resolving gel working solution for 2 x mini Protean 3 1.5 mm gels (Biorad)

- 11.2 ml 7.5% RGP
- Immediately before use 3.75 ml of a 30% Acrylamide stock was added
- Also immediately before use, to polymerise the gel, 150 µl ammonium persulphate and 15 µl TEMED was added

10% Resolving Gel Premix (10% RGP)

- 37.5 ml 1.5M Tris pH 8.8 stock solution

- 1.5 ml 10% SDS

- 60 ml distilled water

10% Resolving gel working solution

- 10 ml of 10% RGP

- Immediately before use 5 ml of a 30% Acrylamide stock was added

- Also immediately before use, to polymerise the gel, 150 µl 10% ammonium persulphate + 15 µl TEMED was added

Stacking Gel Premix (SGP)

- 25 ml 0.5 M Tris pH 6.8 stock solution

- 1 ml 10% SDS

- 60 ml distilled water

Stacking gel working solution for 2 x mini Protean 3 1.5 mm gels (Biorad)

- 8.6 ml SGP

- Immediately before use 1.33 ml Acrylamide was added

- Also immediately before use, to polymerise the gel, 150 µl 10% ammonium persulphate + 15 µl TEMED was added

6x Laemmli Loading Dye

- 60% glycerol
- 300 mM Tris-Hcl pH 6.8
- 12 mM EDTA
- 0.05% bromophenol blue
- 12% β-mercaptaethanol

10x SDS-PAGE running buffer stock solution

- 250 mM Trizma base
- 1.9 M Glycine
- 10% SDS (10 g dissolved per Litre)

1x SDS-PAGE running buffer

- 100 ml 10x SDS-PAGE running buffer stock
- 900 ml distilled water

2.2.9.2 Theory and Protocol for SDS-PAGE

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a method of separating proteins based on their molecular weight through the use of a stacking gel which separates proteins on electrophoretic mobility and resolving gel that separates proteins through the gel based on size. Proteins within a sample are firstly denatured and negatively charged by exposure to a detergent such as sodium dodecyl sulphate (SDS). The amount of bound SDS is relative to the size of the protein and hence proteins will a higher

molecular mass will be more negatively charged to more bound SDS. The SDS bound proteins are then loaded onto a gel matrix through which they are separated in an electric field.

All gels made contained either 7.5% or 10% acrylamide and were made using the buffers listed in section 2.2.9.1 using Mini Protean 3 gel casting apparatus (Biorad).assembled according to the manufacturer's instruction, with spacer plates that were 1.5 mm in thickness. Once gels were set, they were loaded into a gel tank, which was filled with 1x running buffer. Lysates of pre-determined concentration (see section 2.2.8) were aliquoted so that all samples would contain the same amount of protein. 50-100 µg of protein was used in all experiments. 6x Laemmli loading dye was then added to each sample so that the 6x buffer was diluted to 1x (the amount of buffer was calculated appropriately per sample). Aliquoted protein samples were then boiled at 95ºC for 5 minutes to denature the proteins and loaded into the wells of the stacking gel and the gels were subjected to an electric voltage of 150 V-200 V for 60-90 minutes (depending on how much protein separation was required). The gel could then be used for further manipulation.

2.2.10 Western blotting

2.2.10.1 Buffers used for western blotting

10x TBS stock solution (Tris buffered saline), pH 7.4

- 500 mM Trizma hydrochloride
- 1.5 M sodium chloride

1x TBS-T (Tris buffered saline with tween)

- 100 ml 10x TBS stock solution
- 1 mM EDTA
- 0.01% (v/v) Tween-20

10x Transfer buffer stock

- 1.9 M glycine
- 250 mM Trizma base

1x Transfer buffer (per litre)

- 900 ml dH_20
- 100 ml 10x transfer buffer stock solution
- 200 ml methanol (GPR)

Stripping Buffer

- 62.5 mM Trizma hydrochloride pH 6.7
- 2% (w/v) SDS
- 0.7% (v/v) β-mercaptaethanol, which was added to buffer immediately before use

2.2.10.2 Western blot protocol

Western blotting is a method of transferring proteins from an SDS-PAGE gel onto a membrane through application of a current that pulls denatured SDS bound proteins within

the gel matrix, which carry a negative overall charge, towards a positively charged electrode. As the proteins migrate towards the positive electrode, out of the gel, they are "trapped" by a polyvinylidene difluoride membranes (PVDF) membrane that binds to the proteins. Once fixed to the membrane, specific antibodies can be used to detect a protein of interest. Following primary antibody binding, a secondary antibody is added that is conjugated to an enzyme, which can work on a substrate to produce a chemiluminescent signal or alternatively the secondary antibody can fluorescent tag in order to allow detection of the antibody-bound protein.

2.2.10.3 Transfer of proteins to a PVDF membrane

For western blotting, proteins were run on a SDS-PAGE gel, as described in section 2.2.9. Western blotting equipment suitable for mini Protean gels was then used according to the manufacturer's instructions (Bio-Rad). This first step was to set up a "sandwich", which entraps both the gel and the PVDF membrane (Millipore) next to one another. A representation of how the apparatus used to assemble this is shown in figure 2.1. Both the membrane and Whatman paper were cut to the same size as the gel and before assembly the membrane was washed for 20 seconds in 100% methanol to moisten the membrane to allow efficient transfer. These cassettes were then covered in ice cold 1x transfer buffer in a Western blot tank, which also contained an ice block to ensure the tank was kept at a cool temperature. A current of 250 mA was then set for 2 hours for the protein transfer to the membrane to occur.

Figure 2.1 Representation of Western blot module

2.2.10.4 Immunoblot analysis and chemoluminescent detection of proteins

Following transfer, the membrane was blocked for 45 minutes in either 3% BSA dissolved in 1x TBS-T buffer (if later using an antibody to detect phosphorylated proteins) or a solution 5% dried low-fat milk powder dissolved in TBS-T. This ensures that non-specific binding of the antibodies to the membrane does not occur. Membranes were then incubated with specific primary antibodies (see section 2.2.10.5) for a suitable period (generally overnight at 4ºC or 1-2 hours for highly sensitive antibodies). The amount of antibody used was as recommended by the manufacturers and was diluted in 5 mls of 3% BSA or 5% low-fat milk. Following this incubation period, the membrane was washed 3 times in 50mls TBS-T buffer, for 10 minutes in continuous shaking to remove any excess or indiscriminately bound antibody. Next, the membrane was incubated with a secondary antibody. This antibody was goat, mouse or rabbit, depending on the host source of the primary antibody and all antibodies were conjugated to a horse radish peroxidise (HRP) enzyme. The antibody was diluted 1:1000, according to the manufacturers recommendations, in 5mls of 5% non-fat milk and an hour long incubation period followed. The membrane was then subjected to three more 10 minute washes in 50mls

TBS-T buffer with continuous shaking. Enhanced chemiluminescent detection was then followed (using the Pierce Supersignal West chemiluminescent substrate for detection of HRP kit). This kit provides two solutions, which when mixed together form the substrate for which the HRP enzyme (conjugated to the secondary antibody) can catalyse a reaction. This results in a luminescent product being formed. The solutions were added in accordance with the manufacturer's instructions. Detection of chemiluminescence was carried out in a dark room using X-ray film (Kodak) or a Fujifilm Las-100 phospho-imager and images were analysed using Image Reader Las 1000 software.

2.2.10.5 Primary and secondary antibodies used for Western Blot Protocol for detection of specific protein.

ANTIBODY	HOST SPECIES	SUPPLIER
Phospho-Tyk2 (Tyr1054/1055)	Rabbit	Cell Signalling
Phospho-Stat1 (Tyr701)	Rabbit	Cell Signalling
p-Stat1 (A-2)	Mouse	Santa Cruz
p-Ser727-STAT1	Goat	Santa Cruz
Phospho-Stat3 (Tyr705)	Rabbit	Cell Signalling
Phospho-Stat5 (Tyr694)	Rabbit	Cell Signalling
Stat1	Rabbit	Cell Signalling
Stat3	Rabbit	Cell Signalling
Stat5	Rabbit	Cell Signalling
MEK1/2 (Ser217/221)	Rabbit	Cell Signalling
phospho-p44/42 MAP Kinase (Thr202/Tyr204)	Mouse	Cell Signalling
p-JAK1 (Tyr1022/Tyr1023)	Goat	Santa Cruz
CD3-ζ (C-20) – C-terminus	Goat	Santa Cruz
IFN-α/βRα (R-100) – C-terminal amino acids 458-557	Rabbit	Santa Cruz
p-Vav (Tyr 174)-R	Rabbit	Santa Cruz
Vav (H-211)	Rabbit	Santa Cruz

ANTIBODY	HOST SPECIES	SUPPLIER
Slp-76 (H-300) – amino acids 234-533	Rabbit	Santa Cruz
ERK2 (K-23)	Rabbit	Santa Cruz
p-Tyr (PY99)	Mouse	Santa Cruz
Anti-Zap-70 clone 2F3.2	Mouse	Upstate Biotechnology
Phospho-Akt	Mouse	Cell Signalling
MMHAR-2 neutralising mouse monoclonal antibody against humn interferon α/β receptor chain 2 (CD118)	Mouse	PBL Interferon Source
RFP	Rabbit	Chemicon International
GFP-HRP (amino acids 1-238 of *Aequorea Victoria* GFP)	Mouse	Santa Cruz
Anti-mouse, HRP-conjugate secondary Ab	Goat	Santa Cruz
Anti-rabbit, HRP-conjugate secondary Ab	Donkey	Santa Cruz
Anti-goat, HRP-conjugate secondary Ab	Donkey	Santa Cruz

2.2.10.6 Reprobing membranes by "stripping" them

In some cases membranes were used to reprobe with another primary antibody. This is especially important when a membrane has been used to detect changes in a phosphorylaed protein as it ensures that any changes seen are real and not a consequence of inaccurate protein loading. In order to strip the membrane of bound antibodies SDS-containing stripping buffer was used. The membrane was incubated with this buffer at 50ºC for 30

minutes. Ten washes with 50 mls of dH_2O then followed. The membrane could then be blocked again and treated with alternative primary and secondary antibodies in the same manner as described in section 2.2.10.4.

2.2.11 Immunprecipitation

This is a method to determine how an individual protein within a live cell binds to other proteins within a complex over time. Cells were plated onto 10 cm dishes in 10ml of starvation media (see section 2.2.7). 10 x 10^6 cells were used per time point (counted using a hemocytometer). After lysis (as described in section 2.2.7) and protein quantification (section 2.2.8), 2 µg antibody was then added to 2 mgs lysate and placed in continuous rotation at 4°C overnight. After 18 hours, 80 µl of hydrated protein A agarose beads or protein G beads, depending on the antibody manufacturer's instructions (in a 1:1 slurry in 1x PBS) was added to each sample and returned to continuous rotation for a further 4 hours. Cells were then spun for 1 minute at 13000 rpm and washed with ice cold lysis buffer. This wash step was repeated four times. The beads were then dried and protein was eluted from the beads through addition of an equal amount of 2x Laemmli buffer followed by boiling at 95°C for 5 minutes. The supernatant was then resolved on a 10% SDS-PAGE gel. The proteins were then transferred onto a nitrocellulose membrane for 2 hours at 250 mA, blocked with 3% BSA and probed with specific antibodies.

2.2.12 Preparation of cells for confocal microscopy and immunoflourescence

2.2.12.1 Buffers used for immunoflourescence and confocal microscopy

1x PBS pH 7.4 (where indicated the same as shown below was used, but was brought to pH 8.0.using the gradual addition of a 10 M stock of NaOH)

- 10 mM phosphate
- 2.7 mM Kcl
- 137 mM NaCl

1x TBS (Tris buffered saline) pH 8.0

- 50 mM Trizma hydrochloride
- 150 mM NaCl

Paraformaldehyde (PFA) solutions for cell fixation

- 10% (w/v) PFA, pH 8.0 stock solution (kept at -20ºC in aliquots when not in use)
- 4% PFA (v/v) pH 8.0 working solution (made by defrosting and diluting the 10% stock)

10x mounting buffer stock with anti-fade agent (stored at -20 ºC when not in use)

-1% (w/v) 1,4-phenyldiamine pH 9.5 (Fluka)

1x mounting buffer with anti-fade agent (pH 7.5-8.0)

- 1 ml 10x mounting buffer stock
- 9 ml of a solution of 50% glycerol dissolved in 1x PBS

Blocking buffer for immunoflourescence (sterile filtered)

- 3% BSA
- 1% Saponin
- 5% FBS
- The above were dissolved in 1x TBS pH 8.0

Antibody buffer for immunoflourescence (This was used to dilute the antibody during incubation periods and was sterile filtered)

- 3% BSA
- 1% Saponin
- The above were dissolved in 1x TBS pH 8.0

2.2.12.2 Using confocal microscopy to visualise cells

2.2.12.2.1 Starvation of cells on pol-L-lysine coated coverslips

Cells were suspended in 200 µl serum-free starvation media and plated onto poly-L-lysine coated coverslips in 6 well plates. Once the cells had adhered to the coverslip after a 10 minute incubation period at 37°C, a further 1 ml of starvation media was added to each well. The cells were then incubated for 2 hours at 37°C. 6000 units/ml Roferon (Roche) antibody was then added to each coverslip-containing well and cells were incubated again in the presence of stimulus at 37°C for the period of time required. Cells were then immediately fixed following stimulation with 1ml 4% ice-cold paraformaldehyde solution and incubated for 20 minutes at room temperature (25°C).

2.2.12.2.2 Preparation of slides

Cells were then washed three times for 5 minutes using 1x PBS (pH 8.0). The coverslips were then gently lowered onto slides containing 20 µl mounting solution. The coverslip was blotted for excess liquid and fixed to the slide with clear nail varnish. Cells were then visualised under oil immersion using a 63x lens of a Leica TCS SP2 confocal microscope and images were analysed using Leica TCR NT software. GFP-tagged constructs were excited at a wavelength of 488 nm and RFP-tagged constructs were excited at a wavelength of 568 nm using a laser.

2.2.12.3 Using immunoflourescence to visualise cells

Cells were starved on poly-L-lysine cover slips in the same manner as described in section 2.2.12.2.1. After fixation cells were washed two times with 2 ml 1x PBS. 1 ml of 1% Saponin dissolved in 1x TBS pH. 8.0 was then added to each well for 5 mins at room temperature to permeabilise the cells. The cells were then blocked with 1 ml blocking buffer for 15 minutes at room temperature. The coverslips were then washed 3 times with 2 ml 1x PBS (pH 8.0) supplemented with 0.01% saponin to maintain permeability of the cells. The coverslips were then transferred to a 12 well plate so that less of the primary antibody would be required to cover the coverslip. Primary antibody (see 2.2.9.5 for primary antibodies) was diluted in 200 µl antibody buffer at a dilution of 1:100 (generally from 200 µg/ml antibody stocks) and incubated overnight at 4°C. Coverslips were placed back into 6 well plates and washed 3 times with 2 mls 1x PBS (containing 0.01% saponin). Coverslips were then placed back into 12 well plates and a fluorescently tagged secondary antibody was added for only two hours at 4ºC. Anti-rabbit Flour-X was used at a dilution of

1:50 (Amersham) and anti-mouse or anti-rabbit Cy3 (Amersham) was used at a dilution of 1:20. Antibodies were diluted to a 200 µl volume in antibody body. After incubation, cells were washed 3 times in 1x pH 8.0 PBS (with 1% saponin added). Two further washes were then carried out 1x pH 80 PBS without saponin present in order to wash the excess off the cells. The coverslips were then mounted onto slides using 20 µl mounting media per slide and fixed using clear nail varnish around the edges of the coverslip. Cells were visualized using a Leica TCS SP2 confocal microscope under oil immersion using a 63x lens. Images were collected and analysed using Leica TCS NT Software.

2.3 Techniques used to isolate human peripheral blood $CD4^+$ T-cells

All experiments in this section were carried out at the Department of Immunology in collaboration with the laboratory of Prof Arne Akbar. Within the laboratory, Dr. Sarah Jackson aided me with some of the experiments.

2.3.1 Blood donation

Healthy human volunteer blood donors were used in the age range of 25-35 years old for all experiments and blood was extracted by a qualified specialist, using sterile equipment. Between 50 and 200 mls blood were removed. Blood was collected into 9 ml tubes containing heparin beads in order to prevent clotting.

2.3.2 Isolation of peripheral blood monuclear cells (PBMCs) from whole blood

The contents of the 9 ml tubes were emptied and diluted in a 1:1 ratio with Hanks Balanced Salt Solution (HBSS Life Technologies) and then the heparin beads were removed from the solution. 20 ml aliquots of Ficoll (Amersham Biosciences) were poured into 50 ml Falcon tubes and to each, 30 mls of the diluted blood was added carefully in order to create an interface between the two solutions. The tubes were then centrifuged for 20 minutes at 2000 rpm in order to separate the constituents of the blood sample through density separation centrifugation in the high density Ficoll media. This resulted in the PBMCs collected at the interface between the Ficoll and the blood plasma with the erythrocytes being pelleted at the bottom of the tube. The PBMCs were then collected from the interface through careful pipetting and resuspended in a 50 ml volume of HBSS. The cells were then centrifuged for 10 minutes at 1800 rpm. The supernatant was removed and a further 50mls HBSS was added. The cells were centrifuged once more at 2800 rpm for 10 minutes. The supernatant was removed once more and the cells were resuspended in a small volume of HBSS. At this stage the cells were counted using a hemocytometer in order for the correct calculations to be made for the amounts of buffers required for the isolations of $CD4^+$ T cells in the following section.

2.3.3 Isolation of $CD4^+$ T-cells from PBMCs

In order to isolate $CD4^+$ T cells, magnetic cell sorting (MACs) was used (Miltenyi Biotec) in accordance with the protocol described by the manufacturers. The principal of this system is that $CD4^+$ T cells are isolated through negative selection. All non-T cells are indirectly magnetically labelled with, firstly, a cocktail of biotin-conjugated monoclonal

antibodies and, secondly, anti-biotin monoclonal antibodies conjugated to Microbeads. The labelled cell mix is then slowly passed through a magnetic field. The non-labelled $CD4^+$ T cells pass straight through and can be collected whilst the biotin-conjugated cells are attracted the magnetic field and are therefore retained.

The suspended cells are firstly centrifuged for 10 minutes at 1800 rpm. The supernatant is then removed and the cells are resuspended in 40 µl of MACs buffer (1x PBS + 4 M EDTA) per 10^7 cells (calculated from earlier cell count). Secondly 10 µl per 10^7 cells of the biotin-antibody cocktail is added and mixed well. The cells are then incubated on ice for 10 minutes. Next 30 µl per 10^7 cells of MACs buffer is added along with 20 µl per 10^7 cells of the Anti-biotin microbeads. This is then mixed and incubated on ice for a further 15 minutes. 10-20 times the volume of additional MACs buffer is then added and the cells were centrifuged at 1800 rpm for 10 minutes. The cells were then resuspended in 500 µl MACs buffer per 10^8 cells.

The cells are then passed through a pre-washed (with MACs buffer) MACs separator and this is carried out according to the manufacturer's instructions. The cells collected are pure $CD4^+$ T cells. Before these cells were used for experiments, a cell count using a hemocytometer was carried out. Also, a sample of cells was kept in order to obtain the purity through FACs analysis. Purity was above 97% in all experiments.

Cells were then centrifuged at 1800 rpm and resuspended in supplemented RPMI and equal numbers of cells were split into 6cm dishes (one per time point). Since the cells are non-transformed they did not require a starvation period. Cells were left for two hours at 37ºC to recover and equilibriate. After two hours cells were stimulated for the with 1 mg/ml OKT3, 1 mg/ml CD28 with 1 mg/ml OKT3 added at the same time or 500-6000 U/ml

IFNα and incubated for the time period required at 37ºC. Cells were then placed on ice, collected and centrifuged at 4ºC for 5 minutes at 1800 rpm. Media was then aspirated and cells were washed with an 1ml 1x Dulbecco's PBS (Biowhittaker) and centrifuged again at 1800 rpm for 5 minutes. Finally cells were lysed in 50-100 µl ice cold NP-40 containing lysis buffer and centrifuged for 20 minutes at 13 000 rpm as in section 2.2.7. Protein concentration was then determined as in section 2.2.8 and Western blot analysis followed (section 2.2.9).

CHAPTER 3

Investigating the role of Vav at the TCR and IFNAR

3.1 Introduction

Vav1 (herein referred to as Vav) is a hematopoietically expressed 95kDa, multi-domain protein comprising both adaptor domains and a catalytic GEF domain. These multiple domains confer it with the ability to interact with a vast array of proteins. In mature $CD4^+$ lymphocytes, phosphorylation of Vav at the TCR is primarily Zap-70-dependent (Katzav et al., 1994). The SH2 domain of Vav also physically interacts with Y315 of Zap-70 (Katzav et al., 1994, Wu wt al., 1997). Phosphorylation of tyrosine 174 in the acidic region of Vav by PTKs allows enzymatic GEF activity to ensue by allowing the catalytic DH domain to gain access to Rac/Rho protein substrates (Aghazadeh et al, 2000). The recruitment of Vav to the TCR and the interactions that it forms within the early signalling complex formed at this receptor have been well characterised (reviewed in Tybulewicz, 2005). See Figure 1.1. for these interactions and section 1.5.6.6 of the Introduction for more detail. The importance of Vav at the TCR is highlighted by the observations that Vav knockout mice display a number of defects in downstream signalling upon TCR ligation such as impaired MAPK responses, calcium flux, NFAT and NFκB-dependent transcription (Costello et al., 1999).

Vav is not only an intermediate of TCR signalling, but is also required at cytokine receptors e.g. the IL-6 receptor and IFNAR (Lee et al., 1997, Platanias & Sweet, 1994) and other haematopoietic receptors such as the B-cell receptor and Fc receptors (Bustelo & Barbacid, 1992, Darby et al., 1994). Through immunoprecipitation studies, it has already been found that Vav physically associates with both Tyk-2 and Ku-80 in response to IFNAR stimulation in U-266 cell line (Uddin et al., 1997). Previous work in our laboratory by Ahmed et al., 2005, demonstrated a role for Zap70 and Lck in downstream ERK MAPK activation in T

cells. Since Vav interacts with Zap70 at the TCR (Katzav et al., 1994), it is likely it may also interact with this protein at the IFNAR in the T cell line.

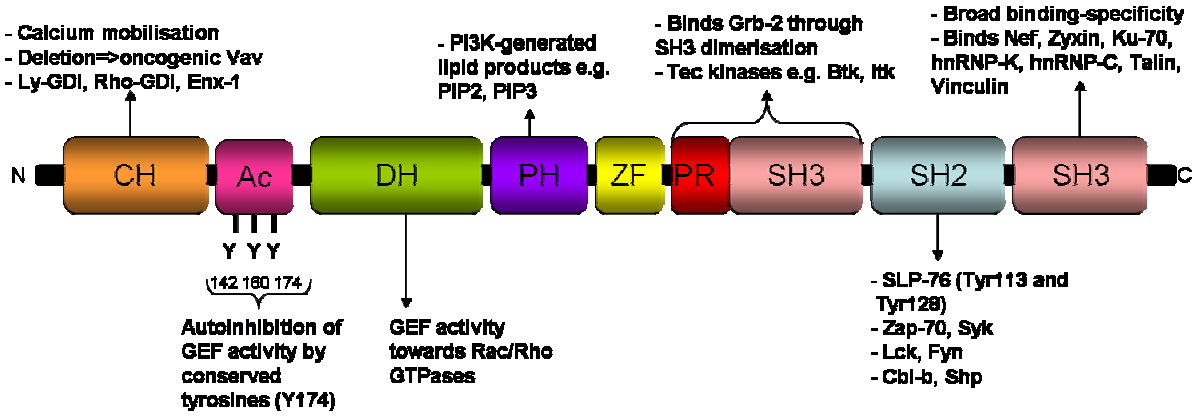


Figure 3.1 Vav domains and interactions formed by each domain

3.2. Aims

In this chapter, the aim was to establish whether Vav is required for IFNAR signalling in the Jurkat T cell line. Experiments are conducted to identify if, like Zap70, its presence is necessary for downstream ERK MAPK. The mode of Vav activation was also investigated to assess whether this mimicked that seen at the TCR.

3.3. Results

3.3.1 Vav is required for ERK1/2 phosphorylation downstream of the TCR and IFNAR upon stimulation

Analyses of the ERK1/2 response in T cells isolated from Vav1 knockout mice upon TCR engagement has shown that the response is diminished in comparison to T cells isolated from wild type mice (Costello et al., 1999). Vav1 deficient Jurkat cells (J.Vav1) have also been created by Cao et al. (2002) through somatic cell gene targeting meaning that Vav expression is completely abrogated in this cell line. In contrast to mouse model studies, previous studies of the ERK1/2 response upon TCR stimulation showed that there was little difference between the phophorylation of ERK1/2 in J.Vav1 cells and Jurkat cells, implying that Vav was not required for this response. However, this work did not verify this as instead it was found that the ERK1/2 response in these cells was actually significantly reduced in comparison to parental Jurkat cells and that this result was reproducible (Figures 3.2a and b). One explanation of the discrepancy is that the initial studies only used cells stimulated at very early time points up to 5 minutes. For the experiments presented here, a longer time course of stimulation of up to an hour was used to draw comparisons. Differences in ERK1/2 phosphorylation in the J.Vav1 cell line were especially noticeable later time points (See Figure 3.2b for a graphical representation).

In order to ensure that any differences in ERK1/2 phosphorylation were genuinely a manifestation of the lack of Vav expression, the expression of other key signalling proteins such as Zap70 and Slp76 were compared (Figure 3.2d and e respectively) to ensure that they

were expressed at equivocal levels through western blotting with the appropriate antibodies. The results confirm that Zap70 and Slp76 expression levels are unaffected.

It has already been established that the IFNAR induces an ERK1/2 response and that for this response to occur, components of the TCR signalling machinery are needed at this receptor such as Zap70, CD45 and Lck (Petricoin III et al. 1997, Ahmed et al., 2005). Investigations were conducted to ascertain whether, as in the case of the TCR, Vav was also required for ERK1/2 phosphorylation at the IFNAR.

Jurkat cells were stimulated over a time course of 30 minutes with Roferon, which is a commercially available, purified IFNα-2 protein. A 30 minute time course was chosen since previous studies in the lab by Ahmed et al. (2005) demonstrated that ERK1/2 phsophorylation drops back to basal levels by 30 minutes. This is a much shorter response than the prolonged response seen at the TCR, which persists for over an hour. J.Vav1 cells were also stimulated for the same time period. J.Vav1 cells that are stably reconstituted with a vector to restore Vav expression to the same level as Jurkat cells (originally generated by Cao et al., 2002) were also stimulated. Jurkat, J.Vav1 and J.Vav1 reconstituted cell line lysates were run side-by-side on an SDS-PAGE gel and Western blotted with anti-phospho-ERK1/2 (See Figure 3.2c). The results show the level of phosphorylation of ERK1/2 remained at basal levels in the J.Vav1 cell line whereas an increase in phosphorylation is clearly observable in both the Jurkat and J.Vav1 reconstituted cell lines. The elevation of MAP kinase activity in Jurkat cells and J.Vav1 cells, which is maximal at 5 minutes, mirrors the time course previously observed in IFNα stimulated cells. The fact that the reconstituted cells display a similar ERK1/2 response to parental Jurkat cells confirms that the absence of

Vav in the J.Vav1 cells is responsible for causing defective signalling since the response is restored upon reconstitution of the Vav gene. To confirm that loading in all lanes was equal and that ERK was indeed present in all cell lines, the blot was reprobed with a total ERK1/2 antibody. These data demonstrate for the first time the requirement for Vav in the IFNAR-induced ERK MAPK response. These data imply that Vav is not only mandatory for ERK MAPK signalling at the TCR, but also at the IFNAR.

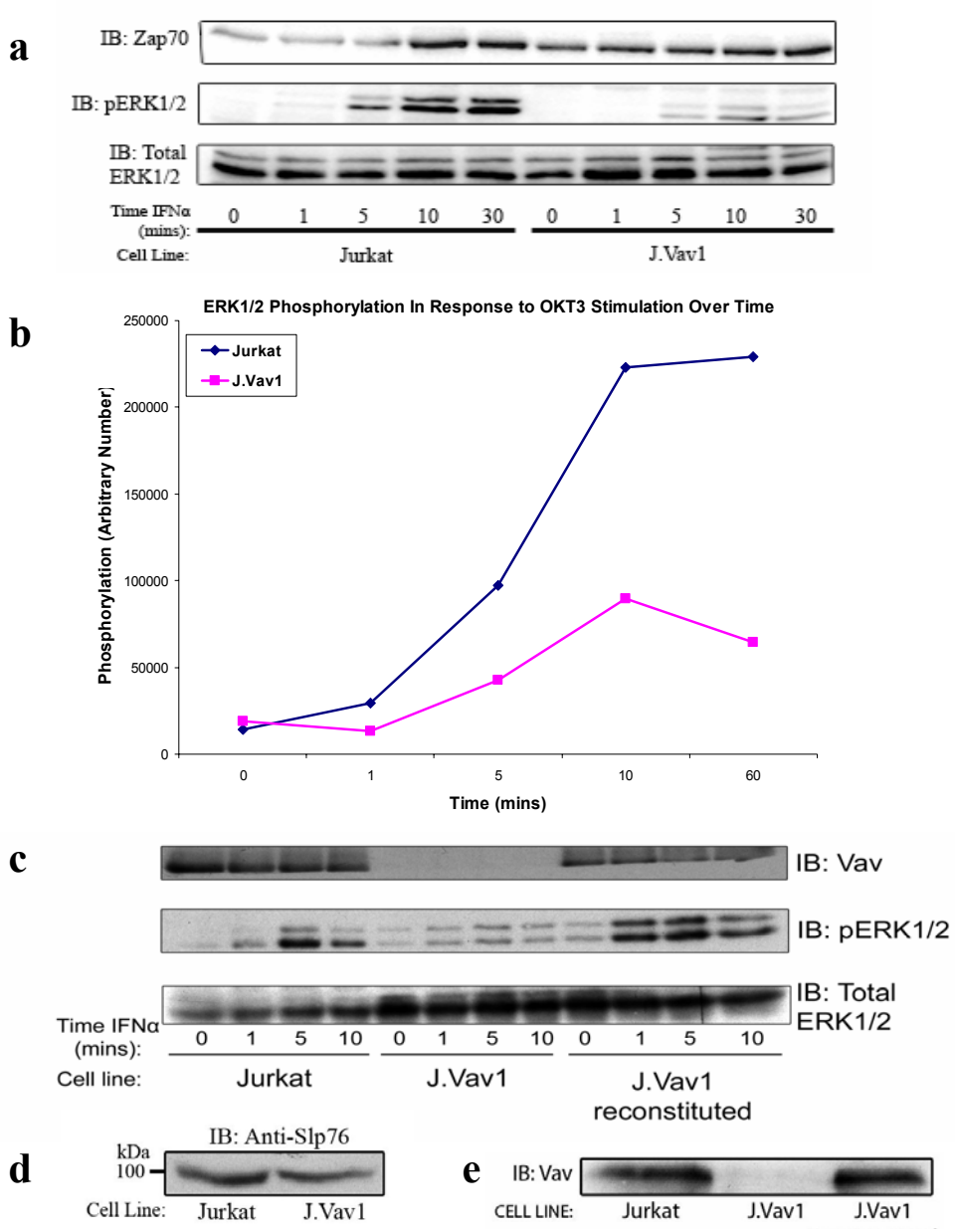


Figure 3.2 ERK is phosphorylated in response to OKT3 and IFNAR

(a) Jurkat and J.Vav1 cells were stimulated with 1 µg/ml OKT3 for the times indicated. Cells were lysed and western blotted with the antibodies indicated. **(b)** The intensity of the phospho-ERK1/2 bands were measured using densitometry and the densities gained were plotted graphically.

(c) Jurkat, J.Vav1 and J.Vav1 reconstituted cells were stimulated with 6000 U/ml Roferon for the times indicated. Cells were lysed and Western blotted with anti-Vav (top panel), anti-pERK1/2 (middle panel). The membrane was then stripped and reprobed with a total ERK1/2 antibody (bottom panel).

(d) and **(e)** show Slp76 and Vav expression respectively in the lysates of the cell lines indicated below the blot

All Western blots shown are representative of three independent experiments.

3.3.2 Vav is tyrosine phosphorylated in response to interferon receptor ligation and this mimics the mode of Vav phosphorylation at the TCR

Vav contains both adaptor modules as well as a catalytic GEF module. To determine whether Vav is actually phosphorylated at the IFNAR in Jurkat cells or whether its role is simply an outcome of its adaptor domains, Jurkat cells were stimulated with IFNα and immunoprecipitated lysates with a Vav antibody, followed by Western blotting with an anti-phosphotyrosine antibody (see Figure 3.4a and c). Vav is phosphorylated within a minute of stimulation, which is similar in kinetics to the time course observed at the TCR as Vav is also phosphorylated within one minute (Zakaria et al., 2004).

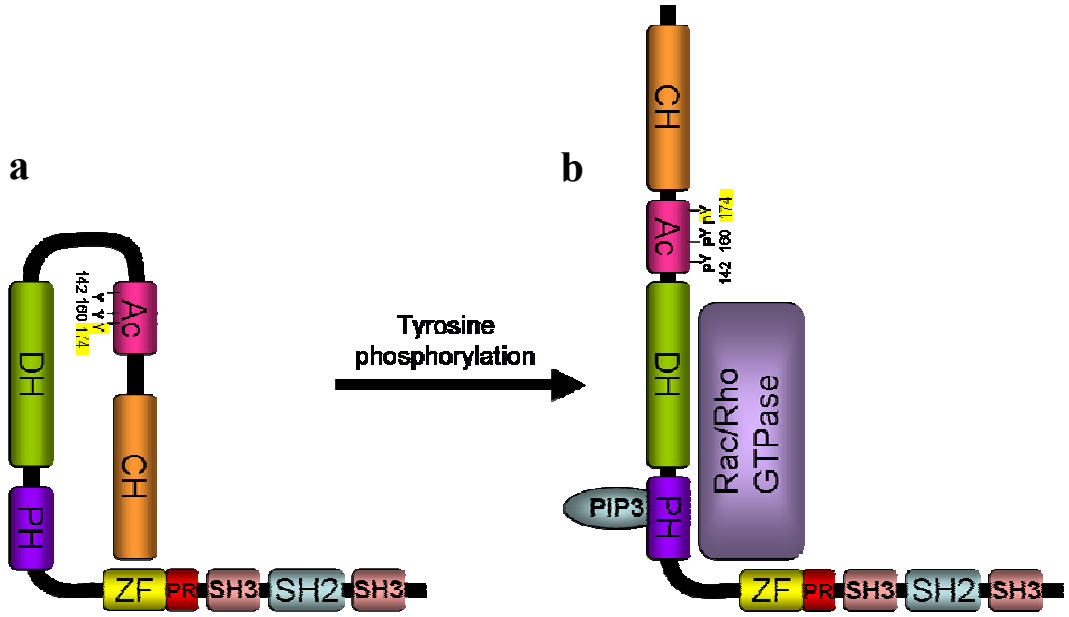


Figure 3.3 Phosphorylation dependent release of the autoinhibitory mechanism at the C-terminus of Vav

(a) The inactive, "closed" conformation of Vav whereby the N-terminus forms an autoinhibitory loop

(b) When Y174 of Vav is phosphorylated, the autoinhibitory loop is disrupted and Vav adopts an "open" conformation. This allows the catalytic DH domain to access its GTPase substrates

Figure adapted from Tybulewicz et al., 2005

At the TCR, Vav is catalytically activated through phosphorylation of Y174, which releases an autoinhibitory loop at the N-terminus that occludes the DH domain (Crespo et al., 1997, Aghazadeh et al, 2000) – See figure 3.3. The protein primarily responsible for this phosphorylation is Zap70 (Margolis et al., 1992). To ascertain whether Vav phosphorylated in a similar fashion to that observed at the TCR, IFNα-stimulated lysates were western blotted with an antibody that specifically detects phosphorylated tyrosine 174 of Vav (Figure 3.4d). To ensure that loading in each lane was equal, the blot was reprobed with a Vav antibody. The results show that Vav is phosphorylated upon this residue within one minute and, surprisingly, that this residue persists in a phosphorylated state even at 30 minutes. It may be the case that phosphorylation of this residue is required for a longer period than the ERK phosphorylation persists in order to mediate downstream cytoskeletal changes through Rac/Rho proteins.

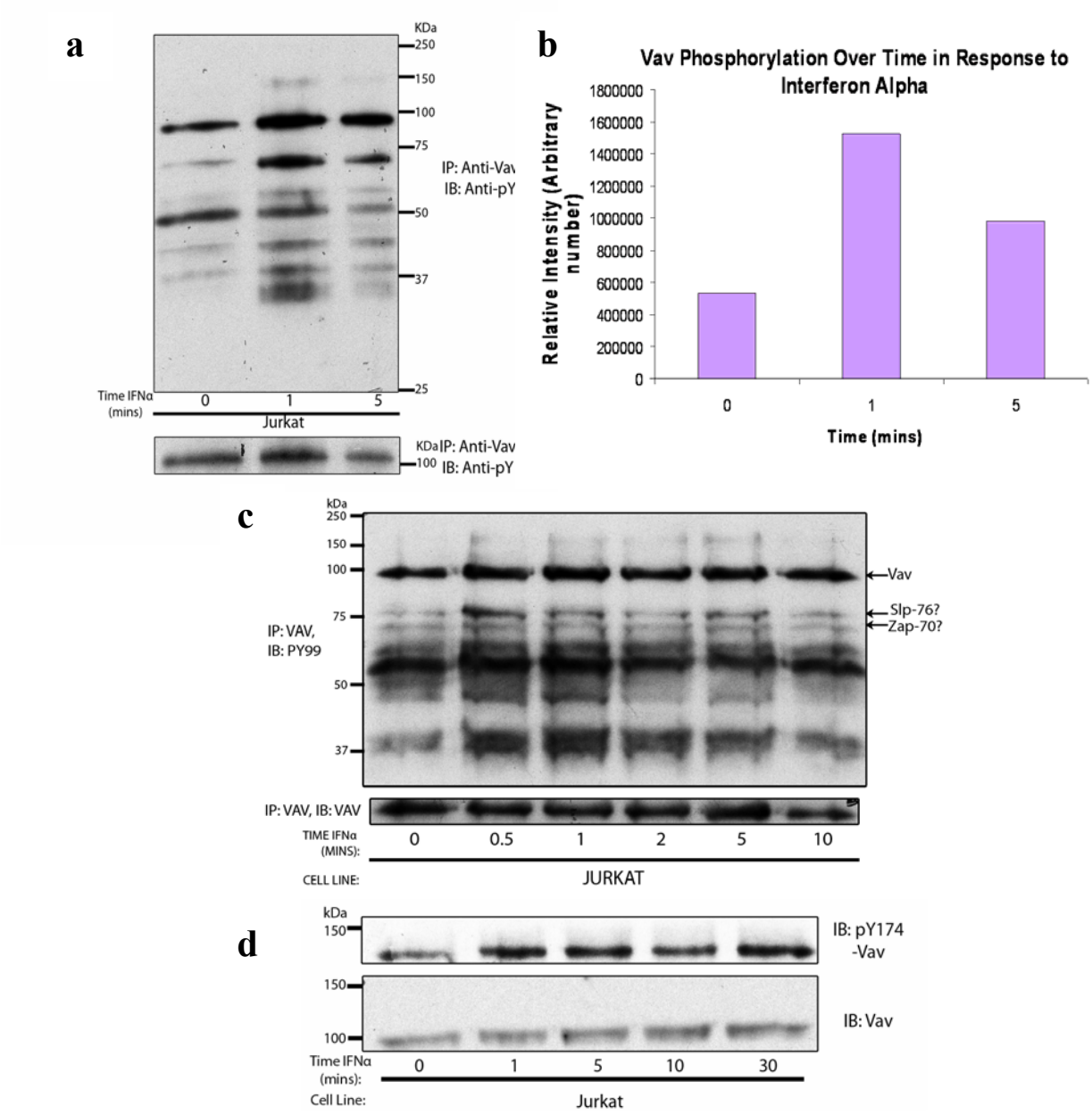


Figure 3.4 Vav is phosphorylated in response to IFNα

(a) and **(c)** Jurkat cells were serum starved and stimulated for the time periods indicated with 6000 units/ml Roferon. Cells were lysed and 2 mg protein was then used to immunoprecipitate with 15 µg anti-Vav antibody. Lysates were then subjected to SDS-PAGE and western blotted with an anti-phospho-tyrosine antibody.

(b) Graphical depiction of intensity of the bands of phosphorylated Vav from blot (a), calculated using densitometry. **(d)** Jurkat cells were serum starved for 2 hours and stimulated with 6000U/ml Roferon. Cell lystaes were then subjected to SDS-PAGE and western blotted with the anti-pY174-Vav antibody (upper panel). To ensure equal loading, the blot was stripped and reprobed with the Vav antibody (lower panel). (a), (c) and (d) are representative of two independent experiments

3.3.3 Zap-70 is required for phosphorylation of Vav at the IFNAR

At the TCR, Zap-70 is phosphorylated by Lck, which in turn phosphorylates downstream substrates such as Slp76 and Vav. The SH2 domain of Vav also physically associates with the Y315 residue of Zap-70 (Katzav et al., 1994, Wu et al., 1996). To confirm whether, as at the TCR, the presence of Zap-70 was necessary to mediate phosphorylation of Vav, the P116 cell line was used, which is a clone of the Jurkat cell line that was found to lack Zap-70 expression (Williams et al., 1998). Both the Zap-70 deficient P116 cell line and Jurkat cells were stimulated with IFNα and the lysates were immunoprecipitated with an anti-Vav antibody followed by western analysis with an anti-phosphotyrosine antibody (Figure 3.5b). The results show that, in the absence of Zap-70, no phosphorylation of Vav is observed whereas Vav phosphorylation is visible in Jurkat cells where Zap-70 was present. This suggests that, as at the TCR, Zap-70 is required for Vav phosphorylation.

3.3.4 Vav phosphorylation does not occur in the absence of Slp76 at the IFNAR

At the TCR, Vav and Slp76 physically associate and co-operate to induce sustained ERK1/2 signalling (Onodera et al., 1996, Tuosto et al., 1996, Wu et al., 1996). J14 cells are a cell line derived from parental Jurkat cells that completely lack Slp76 expression (Yablonski et al., 1998). It is possible that these proteins also interact at the IFNAR and to ascertain whether Slp76 was a requirement for proper Vav phosphorylation, both IFNα stimulated Jurkat and J14 cell lysates were immunoprecipitated with an anti-Slp76 antibody and probed for tyrosine phosphorylated proteins on a Western blot (Figure 3.5a). It was observed that, in contrast to Jurkat cells, Vav phosphorylation was absent in J14 cells. This suggests that an Slp76-Vav interaction occurs and that this is necessary for Vav to be properly

phosphorylated. Since Slp76 is an adaptor protein, it is probable that Slp76 acts to stabilise Vav in a complex so that it is accessible to upstream kinases. Interestingly, the absence of Slp76 in J14 cells seemed to completely abrogate Vav binding partners and only a couple of phosphorylated bands of protein were observed from the J14 cell lysates, even at basal time points. In the Jurkat cell line, bands appeared at approximately 76kDa and 70kDa, which are likely to correspond to Slp76 and Zap-70 respectively yet these bands were absent in the J14 cell line. Since both of these proteins are immunoprecipitated with Vav upon IFNα stimulation, this could indicate that Vav, Slp76 and Zap-70 form a trimeric complex at the IFNAR.

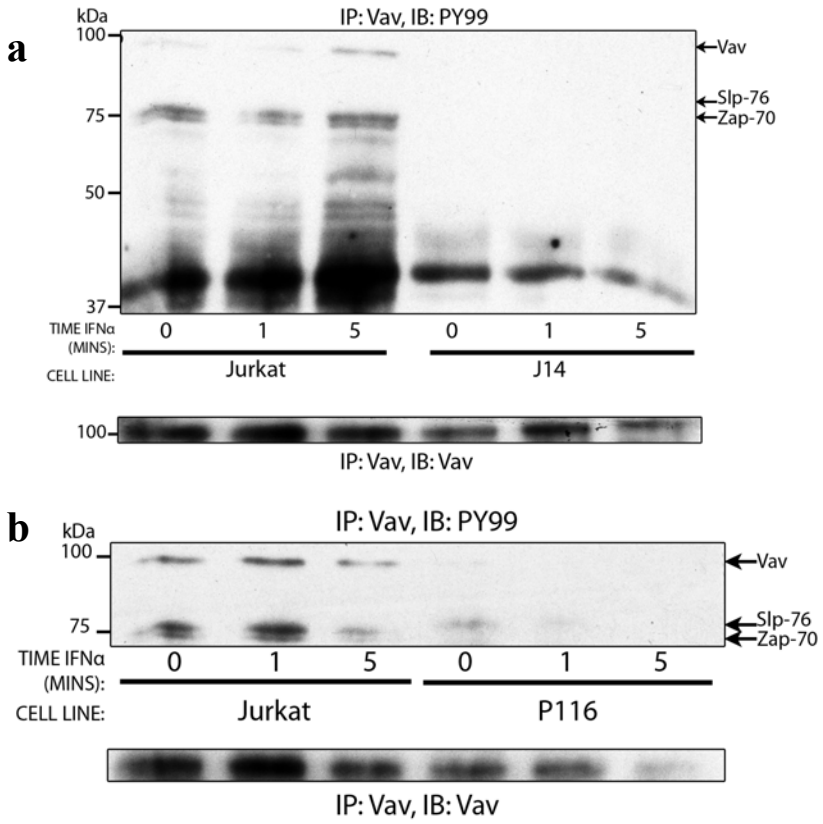


Figure 3.5 Zap-70 and Slp-76 are required for Vav phosphorylation

(a) and **(b)** Jurkat and J14 cells Jurkat and P116 cells respectively were serum starved and stimulated for the time periods indicated with 6000units/ml Roferon. Cells were lysed and 2mgs protein was then used to immunoprecipitate with 15µg anti-Vav antibody. Lysates were then subjected to SDS-PAGE and western blotted with an anti-phospho-tyrosine antibody. 3.5 (a) and (b) are representative of three independent experiments

3.3.5 Localisation studies of Vav through fluorescent tagging

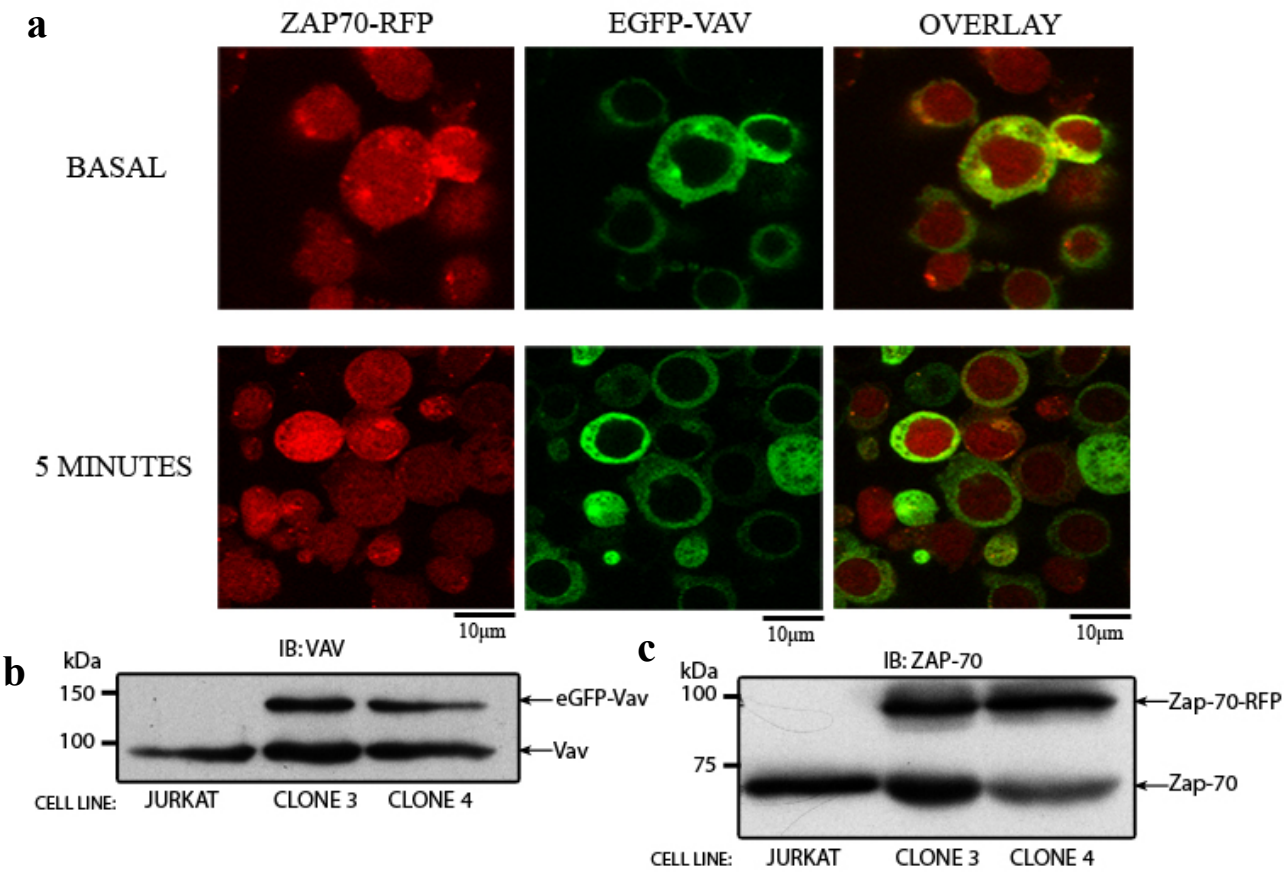
To expand upon the studies in the previous sections experiments were conducted to accomplish visualisation of how IFNα stimulation affects Vav localisation within the cell. To achieve this, reconstitution of J.Vav1 cells was attempted with a vector encoding Vav1 plus an N-terminal eGFP tag. As J.Vav1 cells are G418 resistant, the eGFP-Vav construct was cloned into pcDNAhygro3.1, which is a hygromycin-resistant vector. This would allow selection of cells through use of antibiotics to select cells that express eGFP tagged Vav1 as well as maintaining selection of Vav1 deficiency through addition of G418. The aim was to reconstitute this cell line so that expression levels of Vav1 were equivocal to that of Jurkat cells so that Vav1 redistribution could more reliably assessed without the protein being over expressed. Unfortunately, despite trying different types of transfection protocols including the use of reagents and electroporation, reconstitution of the cells was unsuccessful as the cells did not survive once antibiotic was added and expression levels were very low through only transient transfections. This is most likely due to the fact that J.Vav1 cells already encompass a plasmid to knock out expression of the Vav1 gene and consequently the cells may be unable support transfection with a secondary plasmid. Additionally, the eGFP-Vav construct is very large in size and transfection of larger plasmids is much harder to achieve in comparison to transfection with smaller plasmids.

As transfection of J.Vav1 cells was not accomplishable, the only other alternative was to over-express eGFP-Vav in Jurkat cells. In light of this, Jurkat cells were electroporated with a G418-resistant vector, which encoded eGFP-Vav1. Transfection levels were higher than that achieved in J.Vav1 cells. Cells expressing the transfected construct were selected by culturing them in the presence of hygromycin for 2-3 weeks and expression was checked

through western blotting. A stable pool of cells that expressed eGFP-Vav were generated, but in order to select cells with a higher expression, individual cells were cloned out in 96 well plates and selected clones with the highest expression in order to gain a population of cells with equal protein over-expression. At the TCR, Zap70 is required to phosphorylate Vav and the two proteins physically associate (Margolis et al., 1992, Katzav et al., 1994). Previous work by Ahmed et al. 2005, has already demonstrated that Zap-70 and Lck redistribute from a cytoplasmic to nuclear localisation upon IFNα stimulation. To assess whether Vav co-localised with Zap70, the two stable populations of cells were co-transfected with the Zap70-RFP vector, which had a different antibiotic resistance (hygromycin resistant) in order to allow selection. The cells were again selected for 2-3 weeks and cloned out on 96 wells and two clones were selected that expressed both Zap70 and eGFP-Vav. Figures 3.5b and 5c show the two clones chosen that over-express both eGFP-Vav and Zap70-RFP respectively.

The localisation of eGFP-Vav only and of both constructs together was then visualised. Unfortunately great differences in localisation between basal and longer time points were not observed (figure 3.6a shows basal and 5 minutes of IFNα stimulation). Studies by Ahmed et al., 2005 showed that Zap70 redistributed from a cytoplasmic to nuclear localisation yet my own results did not seem to parallel this and no difference in cellular distribution was observable. This could have been due to the fact that both proteins were over-expressed or cells were not properly stimulated. An additional experiment was also carried out with the hope of visualising changes in the eGFP tagged Vav construct localisation alone within transfected cells, but again discernible differences in localisation were not detected (Figure 3.6d).

In order to try and further elucidate whether Vav localisation differed over time, immunoflouresecence techniques were employed and cells were stained firstly with an anti-Vav antibody followed by secondary labelling with a fluorescently tagged Cy3 antibody. This would allow determination of the localisation of only wild-type protein rather than over-expressed protein (Figure3.5e). Vav staining was successfully and differences in localisation were observable. At the basal time point, Vav was membrane localised, which is agreement with previous observations at the TCR where Vav is found membrane localised and subsequently, upon TCR stimulation, redistributes to membrane localised clusters (Miletic et al., 2006, Salojin et al., 2000). Similar to at the TCR, Vav seemed to cluster by 10 minutes. Had time allowed, repetition and optimisation of these studies would have led to a closer insight into the time-dependent localisation of Vav following IFNAR stimulation.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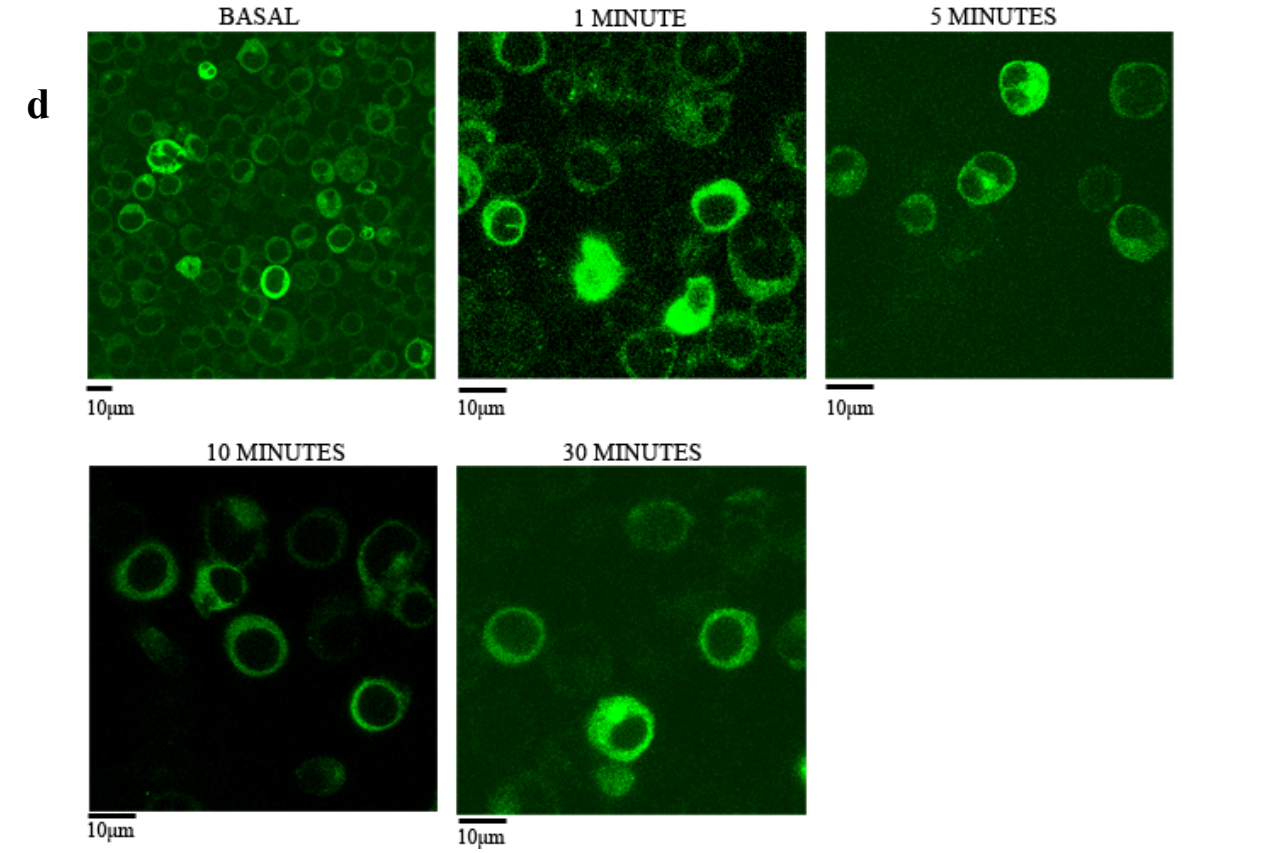
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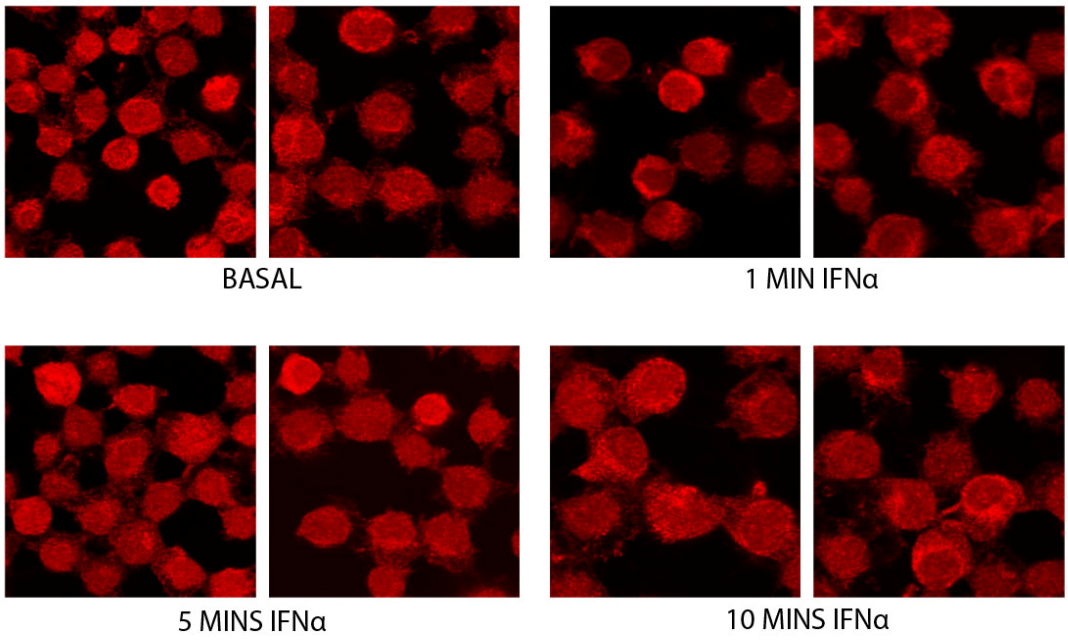


Figure 3.6 Vav localisation in response to IFNα

(a) Jurkat cells bearing eGFP-Vav and Zap70 constructs were plated onto poly-L-lysine coated coverslips, serum starved for 2 hours and stimulated with 6000U/ml Roferon for the time periods indicated. Cells were then washed and mounted and the distribution of Zap70-RFP and eGFPVav was visualised using confocal microscopy. **(b) and (c)** Western blot to show expression of Jurkat cells stably transfected with eGFP-Vav and Zap70-RFP respectively.

Cells stably transfected with eGFP-Vav **(d)** or wild-type Jurkat cells **(e)** were plated onto coverslips and serum starved for 2 hours, followed by stimulation with 6000U/ml Roferon. Cells were then fixed in 4% paraformaldeyde. For panel (d), cells were then washed in PBS and mounted on coverslips and the localisation of eGFP-Vav was visualised using confocal microscopy.

For panel (e), cells were incubated with an anti-Vav antibody overnight, washed, and then incubated with anti-Cy3 antibody for a further two hours. Cells were then washed in PBS and mounted on coverslips. Cy3 tagged Vav was visualised using confocal microscopy.

3.3.6 Effect of Vav mutations upon the downstream ERK1/2 response

At the TCR, large scale mutagenesis and truncation studies have been conducted using 66 mutants to determine the effect of mutations on the structure of Vav and the effect upon downstream signalling (Zugaza et al., 2002), but no studies have been carried out to determine the effect of mutations upon the role of Vav in IFNAR responses. A number of mutants were generated with the intention of establishing the effect of each mutant upon the localisation and signalling by Vav and to establish whether the requirement of specific residues was the same as at the TCR (Figure 3.7a). As mentioned earlier, Vav requires phosphorylation at tyrosine 174 in order to be catalytically active. Therefore an eGFP-Vav mutant construct was produced, bearing an A to T point mutation resulting in a tyrosine to phenylalanine amino acid substation at position 174.

Vav physically interacts with Zap70 through the SH2 domain (Katzav et al., 1994). Another eGFP-Vav construct was also created, bearing an arginine to lysine mutation that would be predicted to disrupt this interaction. The FLAVR sequence of SH2 domains is known to interact with phospho-tyrosines and therefore the arginine at the end of this sequence was mutated. The phospho-tyrosine in Zap70 that interacts with the Vav SH2 domain is located at position 315 (Wu et al., 1997). The Zap70-RFP construct cloned earlier was also used to make another tyrosine to phenylalanine substation at tyrosine 315 as this would also be predicted to disrupt the Zap70-Vav SH2 domain interaction if the two proteins bind to one another in a similar manner to at the TCR (Figure 3.7c). The last mutant created was one bearing a point mutation within the DH domain of Vav at position 213. A point mutation was made from T to G resulting in an amino acid substitution from leucine to glutamine. Previous studies found that this mutation disables the catalytic GEF activity of this domain towards its

downstream substrates (Zugaza et al., 2004). At the TCR, Vav activates downstream ERK MAPK signalling though GEF and GEF-independent pathways. In order to ascertain whether, at the IFNAR, the role of Vav in this pathway was GEF dependent or required only the Vav adaptor domains, a truncated version of Vav composed of only the SH2/SH3/SH2 cassette was also generated by creating primers to amplify this region and cloning into the eGFP vector (Figure3.7b). The intention of creating these mutants and the truncated version of Vav was to reconstitute J.Vav1 cells with them in order to establish whether ERK1/2 signalling was restored and what residues were necessary for this response. As each construct was fluorescently tagged, this would additionally allow visualisation of the location of these constructs using confocal microscopy.

Similarly to the wild-type eGFP-Vav construct used in section 3.3.5, J.Vav1 cells were transfected with the mutant constructs that had been created, but a high efficiency of transfection was not attained. Several protocols for transfection were attempted, but none of these protocols resulted in stable reconstitution of the J.Vav1 cells with any of the constructs. Due to the fact that in Vav in Jurkat cells over-expressing eGFP tagged Vav no changes in localisation of tagged protein could be seen, the mutant constructs were not transfected into Jurkat cells as over-expressing the protein may not give reliable results. Studies using the mutant constructs that I created therefore had to be abandoned.

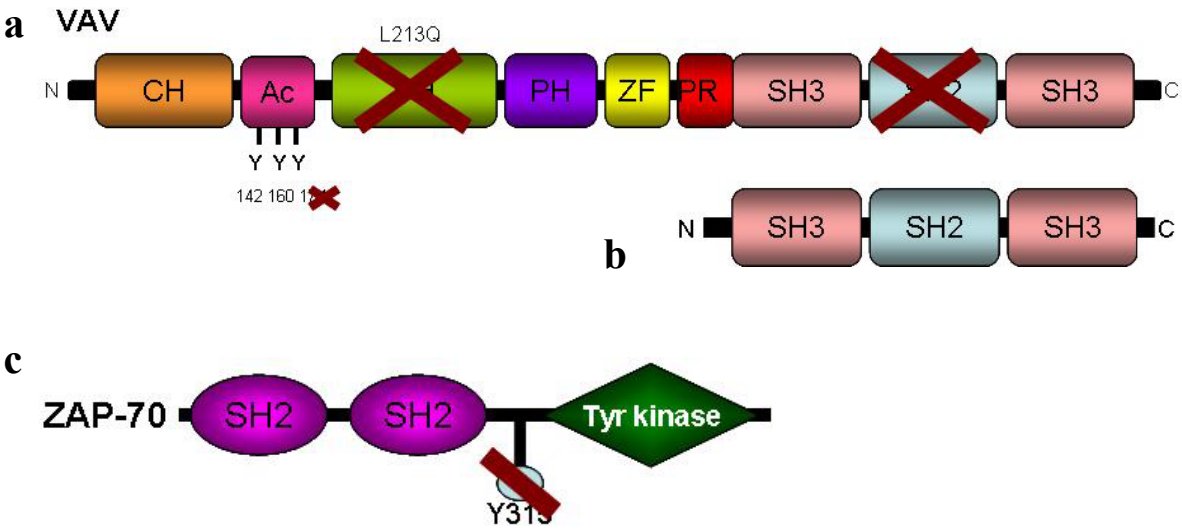


Figure 3.7 Vav and Zap-70 mutants created

(a) Three Vav point mutants were created:- Y174F in the acidic region, L213Q in the DH domain and an arginine to lysine point mutation of the FLAVR sequence of the Vav SH2 domain (R696K)

(b) A Vav truncation was also created bearing the SH3-SH2-SH3 domain cassette only

(c) A Y315F mutant of Zap70 was also created

3.4 Discussion

The results of this chapter clearly define a role for Vav in downstream ERK1/2 phosphorylation in response to IFNα in the Jurkat T cell line. In addition, the mechanism of activation of Vav seems to be similar to that observed upon TCR engagement. Furthermore, Zap70 and Slp76 are required for Vav phosphorylation. Again, this parallels observations at the TCR as both Zap70 and Slp76 bind to Vav and are required for its phosphorylation and recruitment into the TCR early signalling complex. Lastly, preliminary findings suggest that Vav clusters upon IFNAR ligation, which is comparable with observations upon TCR ligation.

3.4.1 Vav requirement in downstream ERK MAPK signalling at the TCR

T cells of Vav1 deficient mice display severely hampered ERK1/2 phosphorylation (Costello et al., 1999). In agreement with these findings in Vav1 deficient mouse T cells, the results presented here show that ERK MAPK signalling is also defective in Vav1 deficient human T cells in response to TCR stimulation with the OKT3. This is shown through comparison of ERK activation in Vav1 positive (Jurkat) and negative T cell lymphoma cell lines (J.Vav1). However this opposes previous findings by Cao et al., 2002 in the J.Vav1 cell line as no defects in this pathway were observed in their report. It is probable that my findings differ as the time course used in my own experiments was longer whereas the previous study had only used a five minute time course. Larger differences in ERK phosphorylation could be seen between the two cell lines from the five minute time point onwards.

There are several reasons why the absence of Vav hampers downstream ERK MAPK signalling. A possible source of this arises from direct Vav-Grb2 binding (Ye & Baltimore, 1994, Ramo-Morales, 1995, Nishida et al., 2001, Ogura et al., 2002) and so Vav absence could disrupt Grb2 stability and thus destabilises its interaction with Sos, which therefore would not be able to effectively initiate the Ras/Raf/MEK/ERK pathway (Buday et al., 1994). In unstimulated cells, Zap70 interacts with SOS, Vav and Grb2 and these interactions are found to be significantly enhanced upon TCR stimulation (Salojin et al., 2000). Also, Sos1 and Sos2 recruitment to LAT is decreased in Vav1-deficient cells, which further affects Ras activation (Reynolds et al., 2004). The absence of Vav in the mouse model has also been found to impinge upon a Grb2 independent pathway that instead results in activation of Ras through a different guanisine nucleotide exchange factor called RasGRP. This exchange factor does not rely upon Grb2-SOS interactions, but instead is activated through its

association with the phospholipid metabolism product, DAG, which is created via hydrolysis of PIP2 by PLCγ. Phosphorylation of PLCγ is reliant upon the GEF activity of Vav as Vav1 negative cells reconstituted with a catalytically inactive form of Vav, mutated in the DH region (L213Q) and truncated at the N-terminal region (Δ1-66), is unable to induce PLCγ phosphorylation and therefore DAG levels are significantly lower leading to a reduction in DAG-dependent RasGRP activation and a reduction in the subsequent ERK MAPK cascade (Zugaza et al.,2004). PLCγ is not regulated directly by Vav, but instead is partly mediated though Rac1-dependent pathways, which is directly subservient towards Vav GEF activation. RasGRP-/- mice infact display a similarly phenotypes to Vav1-/- mice in that the levels of Ras and ERK activation are decreased to a similar level. Both types of knockout mice exhibit a block in the developmental transition from double positive T cells to mature single positive peripheral T cells (Turner & Billadeau, 2002), which is further evidence to support a link between these two proteins. The pathway that links Rac to PLCγ is still not fully expounded, but one route could be through the Rac activated phosphatidylinositol-4-phosphate 5-kinase (PIP5K), which regulates the levels of PIP2. PIP2 is the substrate for PLCγ and so lowered levels would result decreased PLCγ activity (Bustelo, 2000). Vav not only affects PLCγ activation through Rac-dependent means but also affects PLCγ activation through LAT-dependent interactions. Phosphorylation of LAT is hampered in the absence of Vav and therefore the phosphotyrosines that normally bind to PLCγ at positions Y175, Y195 and Y235 of LAT are not created, which further prevents downstream PLCγ regulated DAG generation and activation of RasGRP (Reynolds et al., 2004).

3.4.2 Vav requirement for ERK signalling at the IFNAR

The requirement of Vav in downstream signalling pathways triggered by the IFNAR has never been characterised. Upon IFNα administration, transcription of many ISGs are triggered through JAK/STAT signalling. This pathway is the best characterised pathway triggered by the IFNAR and is also utilised by most other cytokine receptors also (Schindler et al., 1992, Silvennoinen et al., 1993). It has become clear that, although signals transmitted through the Jak/Stat pathway controls transcription of a huge number of ISGs, this pathway is not sufficient to account for all transcriptional changes induced by the IFNAR and other pathways are necessary. One such pathway is the ERK MAPK pathway (Lund et al., 1997, Ahmed et al., 2005). Previous studies have established a role for both Lck and Zap70 in this pathway (Ahmed et al., 2005). Both of these proteins are critical for complex formation by Vav at the TCR (Katzav et al., 1994, Han et al., 1997, Wu et al., 1997, Salojin et al., 2000). Studies herein present the novel finding that Vav is also required for ERK MAPK signalling and in its absence this pathway is virtually ablated.

It would be interesting to determine if, as at the TCR, the role of Vav in this pathway relies upon both GEF-dependent and GEF-independent activation of Ras or whether only one pathway alone is accountable for the role of Vav in this pathway. At the TCR, both pathways are important for Vav-dependent Ras activation, as described in section 3.4.1 (Reynolds et al., 2004). Through GEF-independent mechanisms, Vav constitutively associates with Grb2 (Ye & Baltimore, 1994, Ramo-Morales, 1995, Nishida et al., 2001, Ogura et al., 2002), which is required for Sos1 and Sos2 recruitment and subsequent activation of Ras. Vav is also necessary for RasGRP activation substantiated by the fact that Vav1 deficient cells a redistribution of RasGRP to the cell surface is no longer observed. RasGRP activation relies upon GEF dependent activation of Rac1, which in turn plays a role in downstream PLCγ

phosphorylation. Activated PLCγ in turn generates DAG, which is required for RasGRP activity towards Ras (Reynolds et al., 2004). One method to determine the dominant pathway would have been to use the mutant constructs that I created to transfect J.Vav1 cells stably so that the expression of each construct was equivalent to the expression of wild type Vav in Jurkat cells. The L213Q mutated Vav would be predicted to halt Vav-dependent activation of RasGRP since, at the TCR, this is reliant upon Rac activation through the DH domain as this point mutation has previously been shown to halt the catalytic activity of this domain (Zugaza et al., 2002). Therefore, if the L231Q Vav construct was used to reconstitute J.Vav1 cells and the ERK1/2 phosphorylation level was restored to that of wild type Jurkat cells, it would implicate that DH-dependent effectors do not account for the primary mode of Ras activation. If ERK1/2 phosphorylation was not restored in the presence of this construct, then the Rac-dependent pathway could account for the involvement of Vav in MAPK signalling at the IFNAR. It would also be useful to compare RasGRP distribution in the presence or absence of Vav via a comparison of Jurkat and J.Vav1 cells.

If Vav is involved in ERK MAPK through GEF independent modes, it is likely that it helps recruit Grb2-SOS complexes. At the TCR, Vav constitutively associates with Grb2 through its SH3 domain (Ye & Baltimore, 1994) and so it would be of interest to examine whether this is true at the IFNAR. This could be achieved by looking at co-localisation of fluorescently tagged Vav and Grb2. Alternatively, a Vav construct mutated at the SH3 domain could be created and used to reconstitute J.Vav1 cells. This mutation would be predicted to disrupt Vav binding with Grb2 and therefore if ERK1/2 phosphorylation was dependent on Vav-Grb2 binding, the response would not be restored. Another approach to determine the relevance of Vav-Grb2 interactions at the IFNAR would be to co-transfect

Jurkat cells with fluorescently tagged constructs to analyse whether they co-localise and also FRET could be used to determine whether the two proteins physically interact. Collectively, such experiments would help to understand how Vav is involved in ERK MAPK signalling at the IFNAR.

3.4.3 Vav phosphorylation and interactions at the IFNAR

Vav has previously been shown to physically interact with the Jak kinase, Tyk2, and the nuclear protein, Ku-80, in U-266 cell line through co-immunoprecipitation studies and forms a trimeric complex with these proteins (Uddin et al., 1997). This interaction is kinase dependent as an inhibitor blocks these associations (Adam et al., 1999). Vav also redistributes from a nuclear to a cytoplasmic localisation within 10-30 minutes of IFNα stimulation, shown through cellular fractionation (Adam et al., 1999). Previously Vav was shown to be phosphorylated in the U-266 cell line (Platanias & Sweet, 1993), but phosphorylation of this protein had never been demonstrated in the Jurkat T-cell line. My results show that Vav is phosphorylated in response to IFNAR ligation within 5 minutes (Figure 3.4a to c), but this drops back to basal levels of phosphorylation by 30 minutes. Furthermore this phosphorylation matches that observed at the TCR since Y174 is phosphorylated. Vav has three tyrosine residues within the acidic region of its N-terminus (Tyr142, Tyr160, and Tyr174), which become phosphorylated upon TCR stimulation (López-Lago et al., 2000). Phosphorylation of Vav activates its guanisine nucleotide factor activity, resulting in the exchange of GDP for GTP upon GTPases belonging to the Rac and Rho families (Crespo et al., 1997). The residue that is most important for regulation of GEF activity is Y174 and mutation of this residue to phenylalanine results in enhanced DH

domain activity whereas mutation of the other two tyrosine residues has a much less profound effect (López-Lago et al., 2000). In its inactive state Vav adopts an autoinhibitory conformation, whereby the N-terminus forms a loop that occludes the DH domain from accessing downstream substrates thus rendering Vav catalytically inactive. NMR studies of residues 170-375 have illustrated that Y174 is buried within the autoinhibitory interface at the DH domain and that this residue is also thermodynamically coupled with the PH domain, which suggests that phospholipids may also stabilise Vav activation. Phosphorylation of Y174 results in the auto-inhibitory N-terminal portion becoming unstructured and thus releases the loop thus exposing the catalytic domain, allowing it to catalyse GTP exchange upon target proteins (Aghazadeh et al, 2000). Future research could be focussed on ascertaining whether all three tyrosines within the acidic region of Vav are also phosphorylated at the IFNAR and the effect of mutation of each tyrosine upon downstream pathways such as ERK MAPK signalling could be determined. Furthermore, other reports have suggested that the PH domain of Vav is required for regulating Vav recruitment to the TCR though binding to phospholipid metabolism products and that this domain is also thought to be involved in GEF activity regulation, most probably by stabilising Vav interactions at the cell membrane (Han et al., 1998, Das et al., 2000, Agazadeh et al., 2000). Therefore it would also be beneficial in future to investigate whether this domain plays a similar role at the IFNAR and also how mutating, truncating or deleting other Vav domains affects IFNAR signalling.

In addition, the presence of Zap70 and Slp76 are required for Vav phosphorylation at the IFNAR. In the absence of either protein, Vav phosphorylation was not observed. At the

TCR, Zap70 is mandatory for phosphorylation of Vav (Michel et al., 1998) as well as several of the proteins that Vav associates with such as Slp76 (Bubeck Wardenburg et al., 1996). In P116 cells, no Vav phosphorylation is observed at all when these cells are stimulated through the TCR (Salojin et al., 1999) and this draws a parallel with my own findings at the IFNAR. Zap70 phosphorylates tyrosines 112 and 128 upon Slp76, which are critical for Vav binding to Slp76 and the interaction between Slp76 and Vav is required for augmentation of downstream IL-2 transcription (Motto et al., 1996, Wu et al., 1996, Raab et al., 1997). Zap70 also directly binds to Vav via an interaction involving Y315 of Zap70 and the SH2 domain of Vav (Katzav et al., 1994, Wu et al., 1996). Since Vav phosphorylation was not observed in Zap70 deficient Jurkat cells, it is likely that Vav is also phosphorylated by Zap70 at the IFNAR although further experiments are required to substantiate this and also more research is needed to find out whether Zap70 physically interacts with Vav at the IFNAR in the same manner as at the TCR. Since, at the TCR Vav binds to Slp76, it is possible that Slp76 is also required for recruitment of Vav at the IFNAR and is required for stabilising Vav in a complex. This could account for the fact that Vav is not phosphorylated in the absence of Slp76. Alternatively, Slp76 may help to stabilise Zap70 in a complex during IFNAR signalling and as a consequence, in the absence of Slp76 in Slp76 deficient cells, Zap70 is not able to access Vav as a substrate.

It would be interesting to conduct a more detailed analysis of how Vav/Zap70 or Vav/Slp76 interactions proceed at the IFNAR. In order to achieve this, mutant constructs of Vav could be used such as the SH2 domain mutated Vav construct that I created in order to see if mutations disrupt the interaction of Vav with other proteins such as Slp76 and Zap70 since binding to both proteins at the TCR is mediated through this domain. Also, fluorescence

tagging of Zap70 and Slp76 could be used to ascertain whether they co-localise with fluorescently tagged Vav constructs. Aside from Zap70 and Slp76, additional Vav interacting partners need to be determined at the IFNAR. At the TCR, Vav interacts with an array of proteins through its adaptor domains so it would be useful to study whether Vav interacts with a similar assortment of proteins at the IFNAR. These interactions are described in more detail in section of the 1.5.6.6 introduction. Proteins that dephosphorylate Vav and negatively regulate signalling events at the IFNAR also need to be determined. One possible protein that could down-regulate Vav is Cbl since association with Vav leads to its dephosphorylation and also Cbl targets Vav for ubiquination at the TCR (Miura-Shimura et al., 2003).

3.4.4 Cellular localisation of Vav in response to IFNAR stimulation

Previous fluorescence imaging studies of a Vav-GFP construct transfected into the J.Vav1 Vav-deficient cells have demonstrated that Vav is recruited to microclusters within 5-10 seconds of initial TCR contact with a ligand-coated coverslip. These Vav containing clusters persist at the contact site as the cell adheres and spreads (Miletic et al., 2006). In contrast, GFP tagged Vav bearing a mutation at Y174 is dissipates from the membrane within 5-10 seconds from the membrane, arising from the inability of Y174F Vav to form stable interactions with other TCR-activated signalling molecules at the early TCR complex (Miletic et al., 2006). As reconstitution of the J.Vav1 cells failed in these studies, over-expression of eGFP tagged Vav was used initially to determine any changes in localisation of Vav over time but Vav appeared to remain membrane localised. Due to the fact that Vav was not at a wild-type level, it could have been possible that untagged Vav took part in IFNAR

signalling events primarily meaning that it was hard to detect localisation changes of fluorescently tagged Vav. However, when fluorescently tagged antibodies were used to tag wild-type Vav in Jurkat cells, changes in localisation were noticeable. Vav changed from a membrane bound localisation to a more diffuse cytoplasmic localisation by 5 minutes and some Vav-rich clusters had appeared by 10 minutes. Conducting more detailed analyses of Vav localisation in future is required since the data collected here was only at the preliminary stage. Previous studies of Vav localisation in response to IFNα showed, through use of cellular fractionation techniques rather than fluorescent tagging, that Vav moved to a nuclear localisation by 10 minutes (Adam et al., 2000) and so it would have been interesting to see whether this occurred also in T-cells. Additionally, studies that have explored fluorescently tagged Zap70 and Lck protein localisation at the IFNAR have shown that they both rapidly translocate to the nucleus upon IFNα stimulation for 30 seconds, but then returned to a cytoplasmic localisation by 5 minutes (Ahmed et al., 2005). Further studies of Vav localisation may also highlighted a nuclear localisation of Vav since Vav contains two putative nuclear localisation signals within its amino acid sequence (Katzav et al., 1989).

3.4.5 Other possible roles for Vav at the IFNAR

Interferons not only result in antiviral effects, but also serve an immunomodulatory role and enhance responsiveness to other cytokines. This can bring about a number of cytoskeletal changes. IFNα2 has previously been shown to induce chemokinesis of both $CD4^+$ and $CD8^+$ cells at various stages of differentiation and also enhances T-cell motility by inducing the up-regulation of the LFA-1 and VLA-4 integrins (Foster et al., 2004). This leads to increased ICAM-1- and fibronectin-dependent migration. Vav is an important mediator of actin

cytoskeleton rearrangements due to its catalytic GEF activity towards Rac/Rho GTPases. Vav1 is a GEF for Rac1, Rac2 and RhoG, which are instrumental in regulating filamentous actin, microtubules, T-cell spreading, membrane ruffling and formation of lamellipodia (Tybulewicz, 2005, Bustelo, 2000). At the TCR, Vav is essential for promoting clustering LFA-1, which binds to ICAM-1 upon the antigen presenting cell leading to a stable interaction between the two cells (Hornstein et al., 2004). Therefore, it is likely that at the IFNAR, Vav is involved in similar processes though this has not yet been investigated and it would therefore be interesting in future to investigate this aspect. Additionally, at the TCR, Vav has been found to associate with the GTPase, dynamin2, through its C-terminal SH3 domain and association with this protein helps to mediate reshaping of the cortical actin cytoskeleton. It is thought that dynamin may act as an intermediate between Vav and Rac1 (Gomez et al., 2005). It would be interesting to determine whether dynamin is also involved in IFNAR signalling.

Although a role for Vav has been shown in downstream signalling through the ERK MAPK cascade, future work could be directed towards finding out if the absence of Vav impinges upon any other signalling events at the IFNAR. It is possible for instance that phospholipid metabolism could be affected through Rac-dependent activation of PLCγ, which would be decreased in the absence of Vav. Furthermore, Vav has also been shown to play a role in other MAPK signalling cascades such as the JNK and p38 MAPK cascades at the TCR (reviewed in Bustelo, 2001). Also Vav is necessary for NFκB activation at the TCR since its absence ablates activation of this transcription factor (Costello et al., 1999). The NFκB transcription factor is also activated in response to IFNAR signalling and PI3K, Stat3 and Tyk2 have been shown to be required for this (reviewed in Du et al., 2007). It is possible that

Vav too may play a role in NFκB activation at the IFNAR since it is required at the TCR. Also, at the TCR, Vav has shown to be required for PI3K activation since the Vav deficient DP thymocytes from Vav1 knockout mice display reduced levels of Akt phosphorylation compared to wild type DP mouse thymocytes. PI3K is also activated at the IFNAR and forms a complex with the IRS-1 protein (Uddin et al., 1997) Akt also is activated in response to IFNAR stimulation (Kaur et al., 2008). Therefore, it is possible Vav may be involved in the PI3K/Akt pathway at the IFNAR also.

3.4.6 General Conclusions

In conclusion, the results of this chapter demonstrate that Vav plays an integral role in ERK MAPK signalling events at the IFNAR and forms a complex with other TCR machinery proteins such as Zap70 and Slp76. Future work is now necessary to determine other proteins that Vav binds to at the IFNAR and which domains are required in order for each interaction to occur. It would also be useful to understand to what degree Vav interactions parallel those observed at the TCR.

CHAPTER 4

A Newly defined role for Slp76 in Interferon α Receptor Signalling

4.1 Introduction

Slp76 is a cytosolic, 76kDa, 533 amino acid adaptor protein lacking any catalytic activity. Its expression is restricted to hematopoietic cells only. At the TCR, Slp76 is recruited upon stimulation and helps nucleate a complex between a multitude of proteins. Slp76 comprises three domains that mediate protein-protein interactions; an acidic N-terminal domain, a central proline-rich region and a C-terminal SH2 domain (see figure 4.1). The acidic region of Slp76 contains three tyrosine residues that are phosphorylated in a Zap70-dependent manner (Wardenburg et al., 1996, Raab et al., 1997). These phosphotyrosines provide important docking sites for the SH2 domains of several proteins. The proteins that Slp76 interacts with at the TCR through these phosphotyrosines as well as the proteins it interacts with at the TCR early signalling complex are depicted in figure 4.1.

The binding partners of Slp76 over time at the TCR, and its role in downstream signalling events emanating from the TCR are well characterised. The absence of Slp76 results in defects in many TCR evoked signalling events (Yablonski et al., 1998). In addition, Slp76 has been shown to play a role in Fc receptor signalling in other hematopoietic cell types such as neutrophils and mast cells (reviewed in Wu & Koretzky, 2004), and has been shown to be required for CXCR4 receptor signalling, which is a chemokine receptor expressed on T-cells (Kremer et al., 2003). Therefore the role of Slp76 is not limited to solely TCR signalling. Slp76 is phosphorylated in a Zap70 and Lck-dependent manner and also co-operates with Vav (Wardenburg et al., 1996, Tuosto et al., 1996, Wu et al., 1996, Fang & Koretzky, 1999). All three of these proteins have been shown to be required at the IFNAR (Platanias & Sweet, 1994, Petricoin III et al., 1997, Lund et al, 1999, Adam et al., 2000, Ahmed et al., 2005) so it

is plausible that SLP76 may form identical interactions at the IFNAR during proximal signalling to those observed during TCR-induced complex assembly.

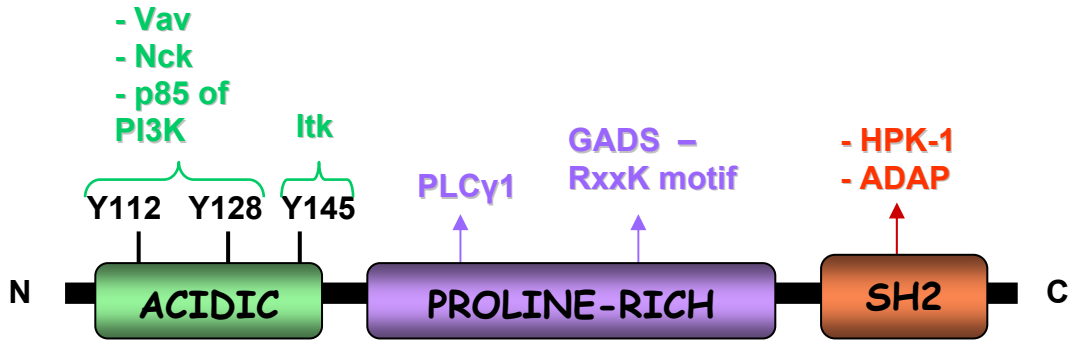


Figure 4.1 Slp76 Domains and proteins that each domain binds

4.2 Aims

The primary aim of the work presented in this chapter was to establish whether Slp76 is a component of IFNAR signalling in Jurkat T cells. To this end, the ERK MAPK phosphorylation was compared in the presence and absence of Slp76. In addition, experiments were conducted to ascertain whether phosphorylation of Slp76 occurs and, if so, whether these phosphorylation events and interactions formed occur in a similar manner to that observed at the TCR.

4.3 Results

4.3.1 Slp76 is required for ERK1/2 phosphorylation downstream of the TCR

Other groups created Slp76 negative mice with the intention of establishing the requirement for Slp76 in downstream signalling at the TCR, but it was found that they exhibit a block in the transition from double negative to double positive T-cells during thymic development. Hence, these Slp76-/- mice display a complete absence of peripheral single positive (CD4+ or CD8+) mature T-cells so signalling could not be studied in mature mouse Slp76 negative T-cells (Clements et al., 1998, Pivniouk et al., 1998).

Since the creation of knockout mice, a Slp76 deficient Jurkat cell line subclone was identified during a screen for TCR-inducible expression of the CD69 activation marker by Yablonski et al. in 1998, which they named J14. This lymphoma cell line allowed studies to be conducted to establish the necessity for Slp76 in mature T-cells, since Slp76 knockout mice yielded a lack of mature peripheral T-cells. The CD69 marker is expressed upon the cell surface of T-cells in a Ras-dependent manner following TCR provocation and its absence therefore suggested J14 cells were defective in the ERK MAPK signal cascade. Indeed, they found that ERK1/2 phosphorylation was diminished in comparison to Jurkat cells.

One aim of this work was to later establish whether the absence of Slp76 also affects IFNAR signalling and to achieve this the J14 cell line was acquired, which was a generous gift from A.Weiss. Firstly it was established whether, as in agreement with previous findings, J14 cells exhibit severely diminished ERK MAPK signalling. To test whether the severity of reduction in ERK MAPK phosphorylation was similar to that observed in Vav deficient J.Vav1 cells, OKT3 stimulated Jurkat, J.Vav1 and J14 cell lysates were run side by side (Figure 4.2a). In

both J.Vav1 and J14 cells the level of ERK phosphorylation was significantly reduced with only a small increase. The level of reduction in both cell lines was very similar (see Figure 4.2b for graphical representation). This shows that upon TCR perturbation, in agreement with previous data, ERK MAPK signalling is severely hampered in the absence of Slp76 compared with wild type Jurkat cells. In order to show that the response could be restored J14 cells were stably reconstituted by transfecting the cells with a Slp76-containing vector, that also contained a C-terminal RFP tag. Cells were stably selected for 2-3 weeks in hygromycin since the vector contained a resistance gene for this. Cells were then cloned out on 96 well plates and a clone was selected that expressed a similar level of tagged Slp76 protein to the level of wild-type Slp76 in Jurkat cells (see Figure 4.3c). Jurkat and J14 reconstituted cells were then stimulated with OKT3 and lysed. Western blotting showed that reconstituting J14 cells restored the level of ERK1/2 phosphorylation to a similar level to Jurkat cells. This proves that it is the lack of Slp76 that causes defective ERK signalling and not another defect in J14 cells. This is further corroborated by the fact that the level of an important Slp76 binding partner, Vav, is expressed at equivalent levels in both J14 and Jurkat cells (Figure 4.2d). In conclusion, the findings of this section agree that Slp76 is imperative for ERK MAPK phosphorylation in response to TCR ligation.

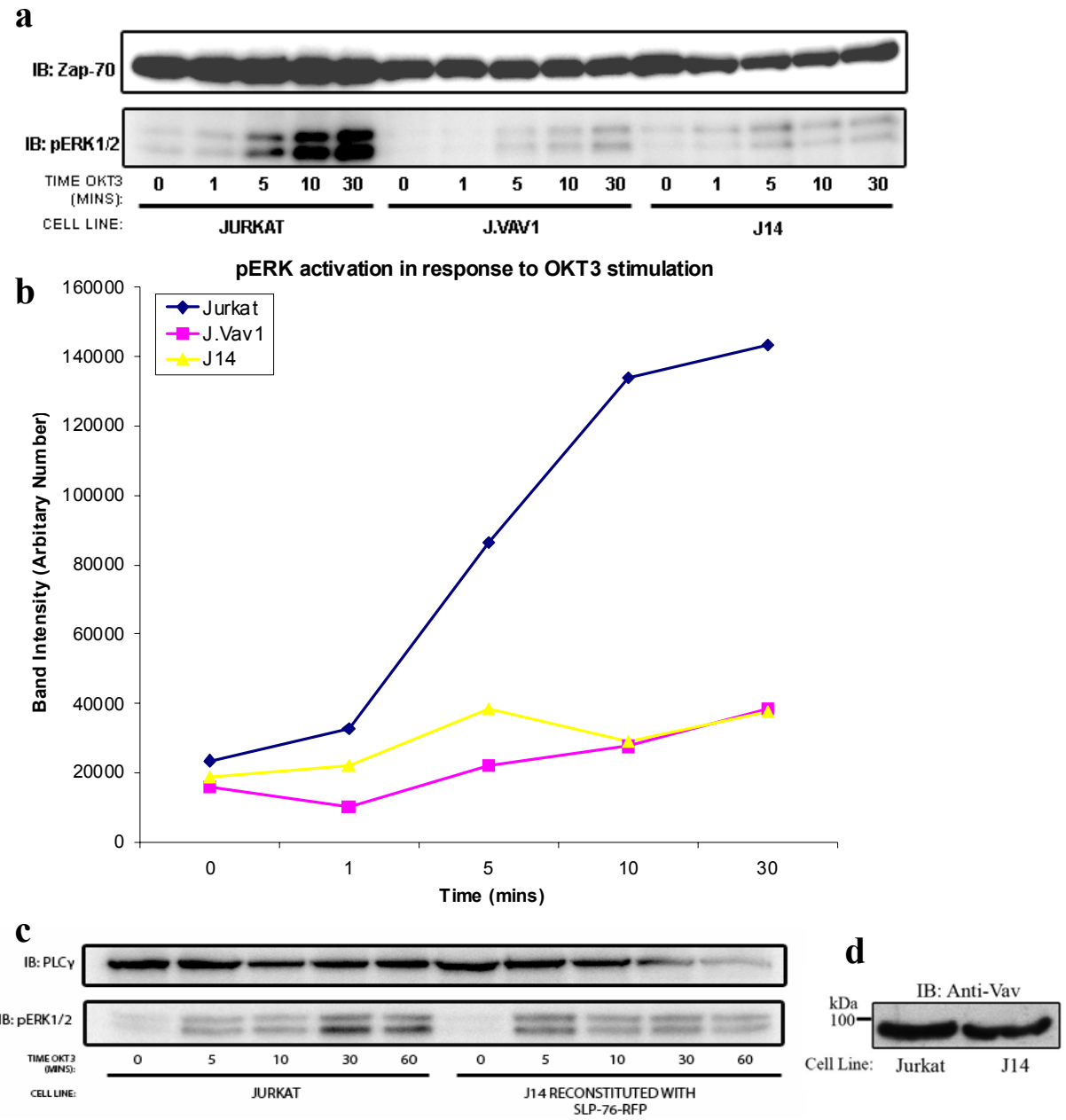


Figure 4.2 Slp76 is required for ERK1/2 phosphorylation in response to OKT3

(a) Jurkat, J.Vav1 (Vav1-/-) or J14 (Slp76-/-) cells were serum starved for 4 hours and stimulated with 1mg/ml OKT3 for the time course indicated. Cells were lysed and separated on an SDS-PAGE gel. Western blot analysis with the antibodies shown proceeded. **(b)** Graphical representation of the phospho-ERK1/2 bands in figure (a), calculated using densitometry. **(c)** Jurkat and J14 cells reconstituted with Slp76-RFP were serum starved for 4 hours, stimulated with 1 µg/ml OKT3 for the time periods indicated, lysed and separated on SDS-PAGE. The gel was then western blotted with the antibodies shown. **(d)** Equal amounts of unstimulated Jurkat and J14 lysates were used for Western blot analysis with the anti-Vav antibody to show that both cell lines express equal amounts of this protein. (a) and (c) are representative of 3 individual experiments

4.3.2 Slp76 is required for ERK1/2 MAPK signalling downstream of the IFNAR

Having verified the necessity for Slp76 at the TCR, it was next investigated whether, like Vav, Slp76 is required for ERK MAPK signalling downstream of the IFNAR. No research has previously been conducted to show a role for this protein at the IFNAR. At the TCR, Vav and Slp76 physically associate and co-operate to induce sustained ERK1/2 signalling (Onodera et al., 1996, Tuosto et al., 1996, Wu et al., 1996) and this co-operation is imperative for IL-2 transcription (Motto et al., 1997, Wu et al., 1996). This interaction is Zap70-dependent (Raab et al., 1997). Since previous studies have ascribed a requirement for Zap70 in IFNAR-induced ERK1/2 signalling (Ahmed et al., 2005) and my own studies have ascribed a role for Vav, it was logical to ascertain whether Slp76 was also a requirement by investigating how the absence of Slp76 in J14 cells affects downstream signalling. Firstly Jurkat, J.Vav1 and J14 cells were stimulated with IFNα over a time course of 30 minutes. Western blot analysis showed that the ERK1/2 phosphorylation was reduced in the J14 cell line (Figure 4.3a) and this reduction was even more severe than that observed in the J.Vav1 cell line, as shown graphically in Figure 4.3b. The next step involved establishing whether reconstitution of J14 cells with Slp76 restores the defective ERK1/2 MAPK signal to a level comparable to Jurkat cells. J14 cells stably reconstituted with Slp76-RFP construct, J14 cells and Jurkat cells were all stimulated with IFNα. Lysates were Western blotted and probed with anti-phospho-ERK1/2. Figure 4.3c shows that an ERK1/2 phosphorylation is clearly detectable in the reconstituted cells and the pattern of phosphorylation mirrors that seen in Jurkat cells as it increases by the 5 and 10 minute time points, but falls back to a basal magnitude by 30 minutes. However, it is worth noting that the basal level of ERK1/2 activity in Slp76-RFP reconstituted cells is higher than in Jurkat cells. Collectively, the data

represented in this section illustrates for the first time, that the absence of Slp76 ablates the downstream ERK1/2 MAPK pathway normally seen in Jurkat cells upon IFNα stimulation.

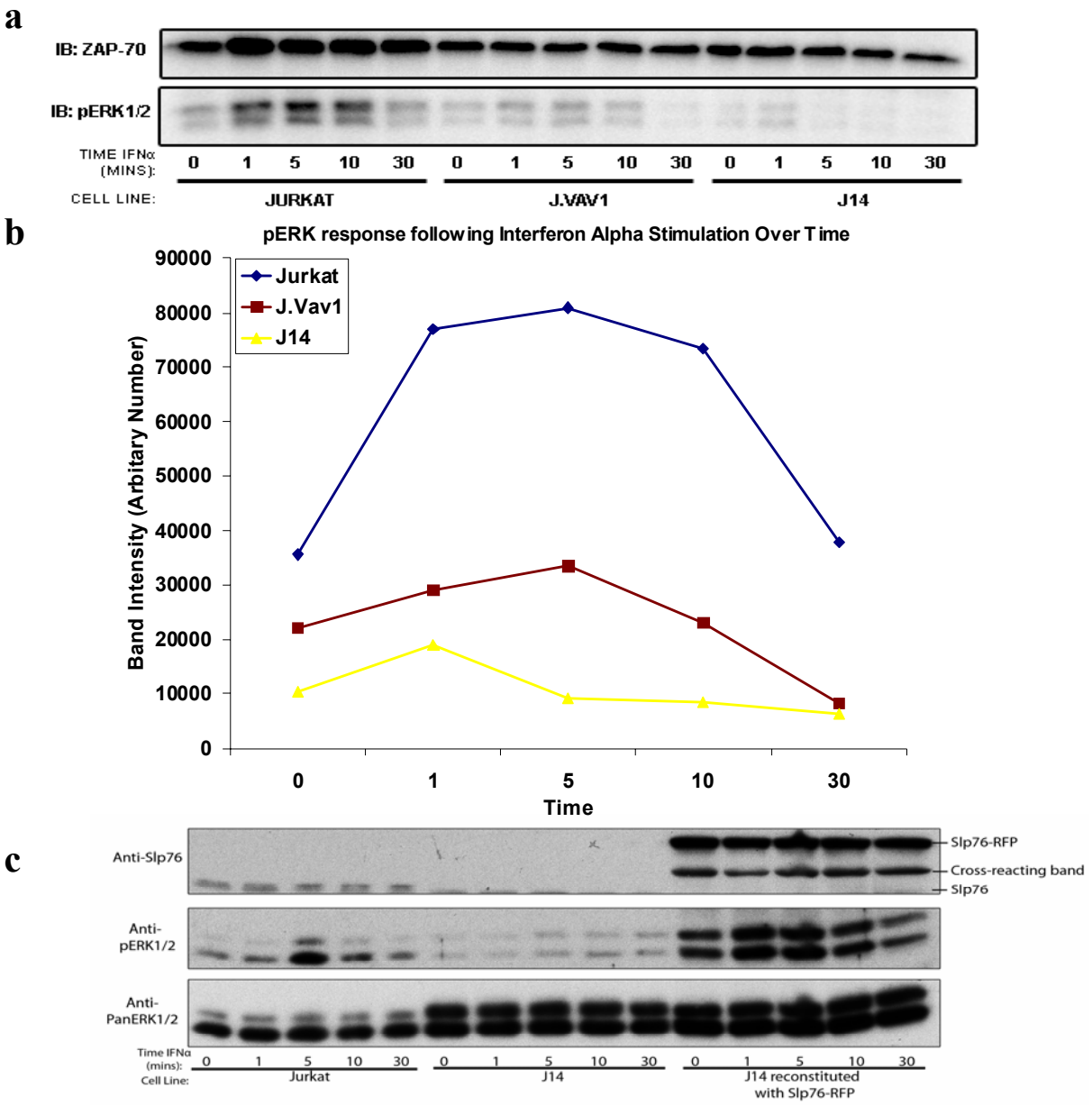


Figure 4.3 Slp76 is required for ERK MAPK phosphorylation in response to IFNα

(a) Jurkat, J.Vav1 and J14 cells were serum-starved for 2 hours then stimulated with 6000 U/ml Roferon for the time periods indicated. Lysates were resolved through SDS-PAGE and western blotted with the antibodies indicated

(b) Depiction of the intensity of pERK1/2 bands on the bottom panel of (a) using densitometry

(c) Jurkat, J14 and J14 reconstituted cells were stimulated for a time course of 30 minutes with 6000 U/ml Roferon. Lysates were resolved through SDS-PAGE and western blotted with the antibodies indicated. 4.3 (a) and (c) are representative of three independent experiments.

4.3.3 Slp76 is phosphorylated in response to Interferon α

At the TCR, Slp76 is phosphorylated upon three key tyrosine residues that reside in the N-terminal acidic region of Slp76. These phospho-tyrosines provide docking sites for the SH2 domains of a number of interacting partners such as Vav, Nck and the p85 subunit of PI3-K. It was investigated whether Slp76 was also phosphorylated in response to IFNα and what proteins Slp76 associates with. Jurkat cells were stimulated with IFNα for a time course of 5 minutes and immunoprecipitated with anti-Slp76. The lysates were then resolved through SDS-PAGE and Western blotted with anti-phosphotyrosine. Figure 4.4a shows that Slp76 phosphorylation is barely detectable at basal levels, but increases significantly upon IFNα administration. Densitometry analysis of the bands of Slp76 protein verifies that Slp76 phosphorylation is enhanced upon IFNα addition (Figure 4.4b). The anti-Slp76 immunoprecipitation was repeated using IFNα-stimulated lysates from a time course of up to 10 minutes to establish whether Vav and Slp76 physically interact over time. Firstly a western blot was probed with anti-phosphotyrosine (Figure 4.4c upper panel). This showed, again, that the level of Slp76 phosphorylation clearly increases upon IFNα stimulation. Another band of protein was also visible with longer exposure of 100kDa size, which could correspond to Vav. In Figure 4.4a, the band was only faintly visible due to shorter exposure. In order to verify whether this protein was indeed Vav, the blot shown in Figure 4.4c was stripped and reprobed with both anti-Slp76 to ensure equal loading of proteins and also anti-Vav (see the bottom panel). The band gained from reprobing with anti-Vav matched the band of phosphorylated protein at 100kDa seen in the top panel therefore proving that phsophorylated Vav and Slp76 physically interact.

To summarise, this section shows for the first time that Slp76 is phosphorylated at the IFNAR and furthermore, similar to observations at the TCR, Slp76 interacts with Vav.

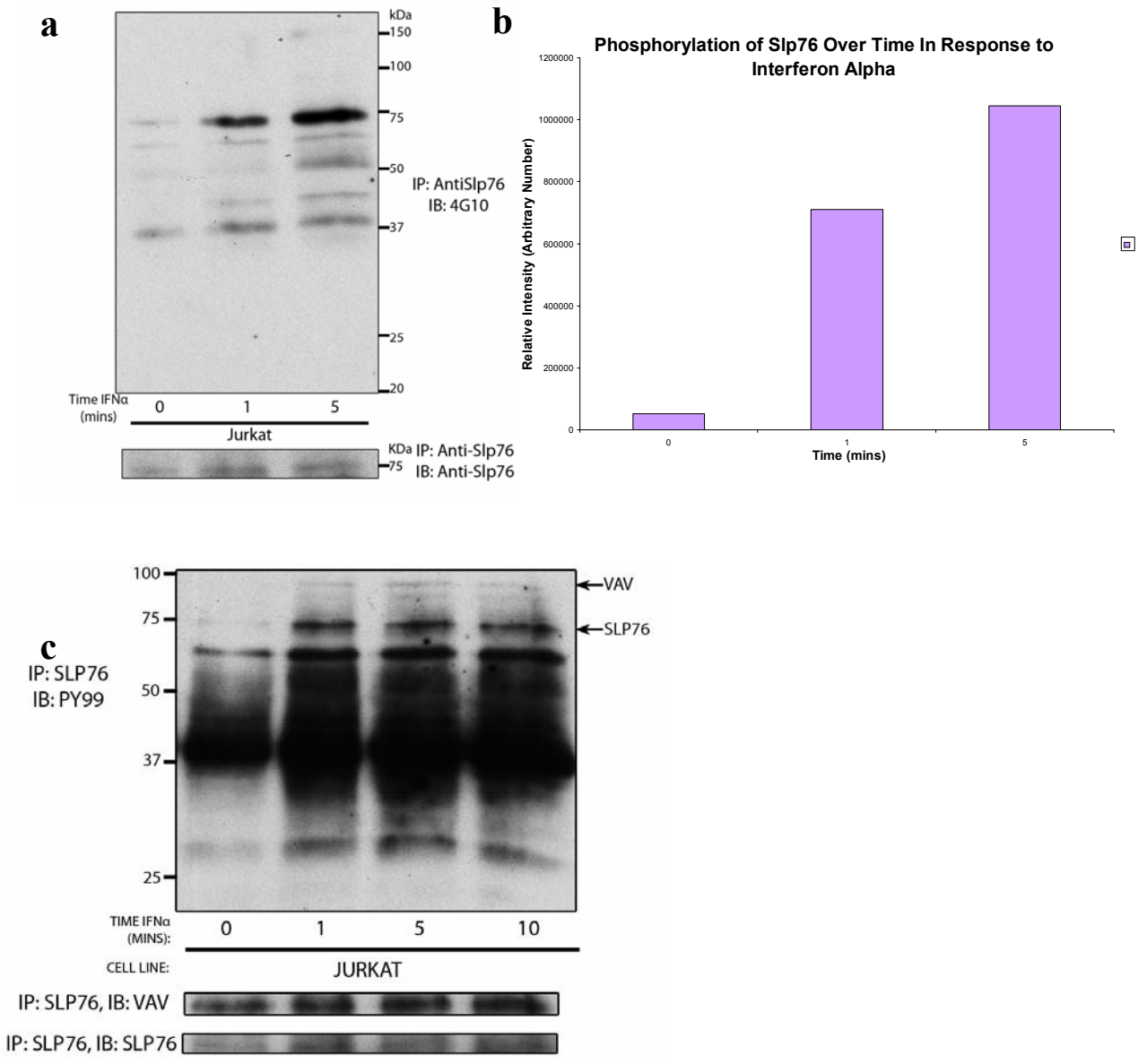


Figure 4.4 Slp76 is phosphorylated in response to IFNα

(a) and **(c)** Cells were serum starved for 2 hours and stimulated with 6000 U/ml Roferon for the time periods indicated. Cells were lysed and immunoprecipitated with anti-Slp76. Lysates were then resolved through SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody (upper panel). The blots were then stripped and reprobed with the antibodies indicated (bottom panel).

(b) Graphical representation of the intensity of Slp76 phosphorylation in blot (a), calculated using densitometry. (a) and (c) are representative of two identical experiments.

4.3.4 Tyrosine phosphorylation of Slp76 is necessary for ERK MAPK signalling downstream of the IFNAR

At the TCR, a functional hierarchy in the N-terminal Slp76 phosphotyrosines exists. Fang et al., 1996 originally identified three tyrosine motifs in the N-terminal region; two DYESP motifs and one DYEPP motif. They found that the tyrosines within the two DYESP motifs (Y112 and Y128) were phosphorylated following TCR engagement and that overexpression of a construct bearing point mutations at both residues significantly affects the ability of Slp76 to promote T cell activation by reducing NFAT activation. These two tyrosines bind to Vav (Wu et al., 1996). Mutation of Y145 within the DYEPP site had an even more profound effect on NFAT promoter activation. Double and triple mutations had a much greater impact on NFAT activity and the reduction was much greater than only single mutants. Since these over-expression experiments, a second paper was published detailing the use of single, double and triple mutants transfected into the J14 cell line at a level of expression that matches that seen in Jurkat cells (Jordan et al., 2006). This meant that the contribution of individual tyrosine residues to signalling cascades that Slp76 participates in could be assessed more thoroughly as the previous transient over-expression experiments were more limiting and less precise as wild type Slp76 was still present. Single tyrosine to phenylalanine muatations at tyrosines 112, 128 and 145 were created and also double mutants and a triple mutant. It was found that, in contrast to the over-expression experiments tyrosine 145 was only phosphorylated in the presence of tyrosines 112 and 128 in response to TCR engagement, as when both were mutated Y145 was not phosphorylated. It was also found that mutation of individual tyrosines reduced ERK1/2 phosphorylation upon TCR stimulation, but the reduction was relatively slight. The greatest reduction for a single

mutation arose when tyrosine 145 was mutated. Cells bearing double mutants resulted in more noticeable impediments in this pathway and cells transfected with the triple mutant exhibited virtually no detectable ERK1/2 phosphorylation (See Figure 4.5b). This highlighted the necessity for Slp76 phosphorylation at the TCR in order for downstream ERK MAPK signalling to ensue. Additionally, it was found that tyrosine 145 was necessary for PLCγ phosphorylation through since J14 cells trasfected with any mutant Slp76 construct with a mutation at this residue displayed significantly reduced phospho-PLCγ compared to wild type Jurkat cells. Similarly mutation of tyrosine 145 also had the most profound effect upon calcium signalling.

Similar to Jordan et al., the contribution of individual tyrosine residues upon Slp76 towards IFNα-induced ERK1/2 phosphorylation was scrutinised. Using site-directed mutagenesis, a total of seven constructs were created bearing one or more tyrosine to phenylalanine substitutions by point mutating an A base to a T within the codon. Single, double and triple mutants of all three tyrosine residues in the acidic region at positions 112, 128 and 145 were generated and sequenced. Each construct was then individually transfected into Slp76-deficient J14 cells to reconstitute the cells with only the mutant form of RFP-tagged Slp76 (see Figure 4.5a for diagram of all constructs). Once a sufficient transfection level was gained, cells were cultured for 2-3 weeks in the presence of hygromycin to select for cells expressing the construct to gain a stably expressing population of cells.

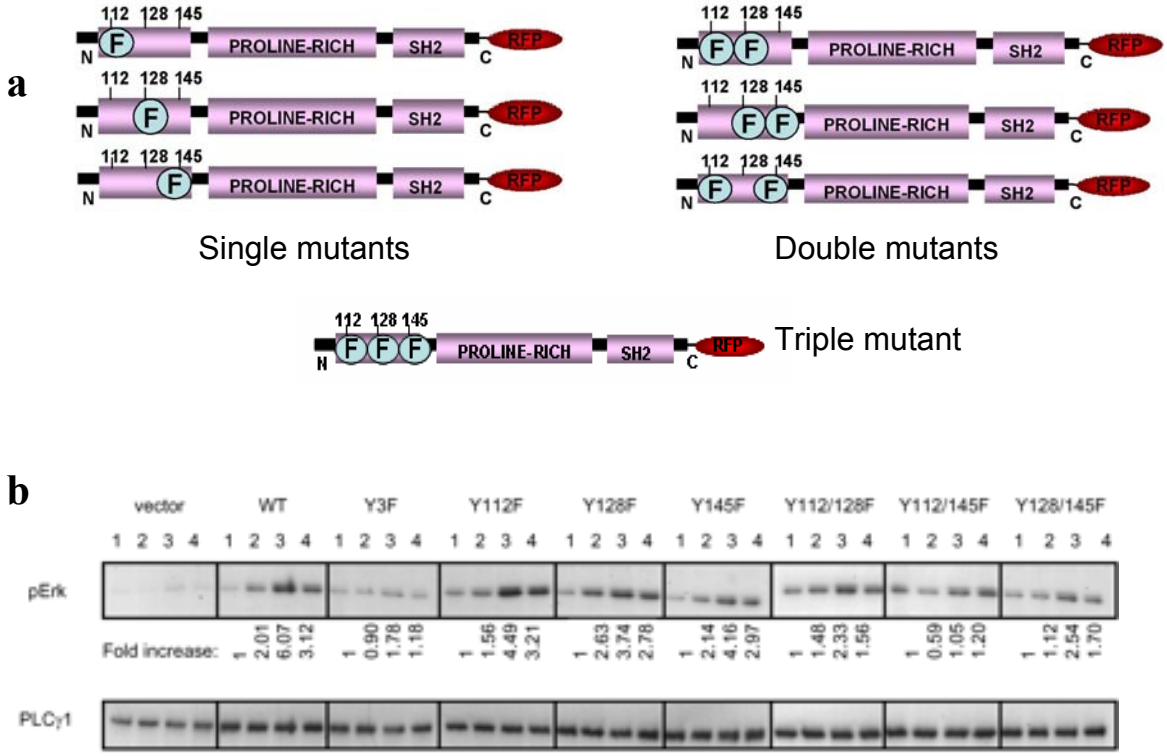


Figure 4.5 Mutant Slp76 constructs created and previous data on effect of mutations on ERK phosphorylation at the TCR (taken from Jordan et al., 2006)

(a) Diagrammatic representation of the Slp76-RFP mutant constructs generated bearing single, double and triple tyrosine (Y) to phenylalanine (F) mutations. All seven constructs were sequenced to check for the appropriate muations and subsequently transfected into J14 cells.

(b) Western blot extracted from a previous report by Jordan et al., 2006 showing the effect of tyrosine to phenylalanine Slp76 mutations on downstream ERK1/2 phsophorylation at the TCR. J14 cells stably expressing the mutant constructs and Jurkat cells were stimulated with OKT3. Lysates were western blotted with anti-phospho-ERK1/2 (upper panel) and anti-PLCγ (lower panel) in order to ensure equal loading

In a bid to resolve whether the role of Slp76 in IFNAR-induced ERK1/2 MAPK signalling was dependent on phosphorylation of the N-terminal tyrosine residues, J14 cells expressing the 3YF triple tyrosine to phenylalanine mutant were stimulated with IFNα to see whether ERK1/2 phosphorylation was restored. Figure 4.6a shows that ERK1/2 phosphorylation is

not restored in the J14 cells bearing the 3YF Slp76 mutant and the level of ERK phosphorylation resembles that seen in the J14 Slp76-deficient cell line. However, an increase in ERK1/2 signalling is clearly observable in wild type Jurkat cells. This shows that the phosphorylation of Slp76 is necessary for downstream ERK1/2 signalling at the IFNAR to occur.

To establish the individual contribution of each tyrosine residue, all of the J14 stable cell lines expressing the single and double Slp76 tyrosine to phenylalanine mutants were stimulated with IFNα for 5 minutes and compared with Jurkat cells stimulated for the same duration (Figure 4.6b)., All of the Slp76 mutant bearing J14 cells displayed lower levels of ERK1/2 phosphorylation when compared with the level of ERK phosphorylation in Jurkat cells. However, it was difficult to determine whether one particular mutation had a more profound effect on ERK1/2 phosphorylation. A slight increase in ERK1/2 phosphorylation was seen when only Y112 or Y128 were mutated. For all other mutants, there was no deviation from basal phospho-ERK1/2 levels upon IFNα administration. This preliminary data shows that three intact Slp76 tyrosine residues are required for MAPK signalling downstream of the IFNAR. However, it is worth noting that the results in Figure 4.6b are representative of a stable pool of cells so expression levels between all cells is not equal.

These data illustrate that Slp76 phosphorylation is imperative for ERK1/2 phosphorylation to be brought about downstream of the ligated IFNAR. This is in agreement with findings by Jordan et al., 2006, who showed that the role of Slp76 at the TCR in this cascade is also phosphorylation dependent (Figure 4.5b).

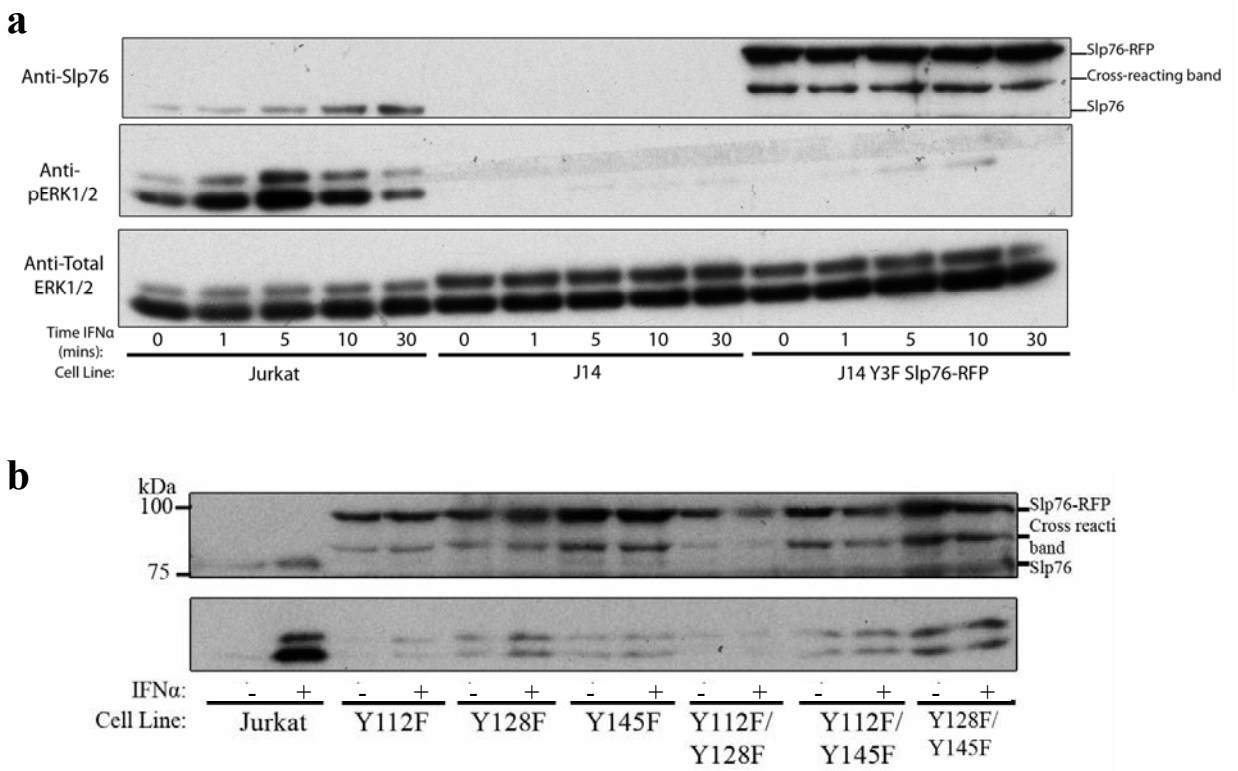


Figure 4.6 The requirement of Slp76 in ERK MAPK signalling downstream of the IFNAR is dependent on three intact tyrosine residues within its N-terminus

(a) Jurkat, J14 and J14 cells reconstituted with a 3YF Slp76-RFP mutant were serum starved for two hours

(b) Jurkat cells and J14 cells stably reconstituted with either single Y-F Slp76-RFP mutants or double Y-F Slp76-RFP mutants were serum starved for 2 hours and were either left unstimulated or stimulated with 6000U/ml Roferon for 5 minutes. Equal amounts of lysates were resolved on an SDS-PAGE gel and Western blotted with ani-phospho-ERK1/2 (lower panel) and anti-Slp76 (upper panel).

4.3.5 Slp76 forms clusters upon IFNα stimulation

Many reports have detailed the changes in localisation of fluorescently tagged Slp76 in response to TCR stimulation. Studies by Bunnell et al., 2002, showed through the use of a number of fluorescently tagged constructs that Slp76 is initially recruited to TCRβ rich clusters that also contain GADs, LAT, Grb2, Cbl and ZAP70 and this occurs within a 15

second time frame post-stimulation. These clusters continually assemble and disassemble. Zap70 stably persists in clusters at the cell surface for up to 20 minutes, but LAT departs from the clusters after a few minutes and Grb2 and Gads dissipate into the cytoplasm. In contrast, Slp76-rich clusters behave uniquely from the other TCR signalling proteins and have been shown to translocate radially along microtubules 1-3 minutes after TCR stimulation to peri-nuclear structures that do not stain for endosomal markers (Bunnell et al., 2002). The initial Lat and Slp76 clusters co-localise and furthermore, recently it has been shown that PLCγ is recruited to clusters, which co-localise with LAT and Slp76-containing clusters (Braiman et al., 2006), supporting the notion that the three proteins form a complex upon TCR engagement.

More in depth studies have since revealed that mutating the three tyrosine residues of Slp76, mutating the GADs-binding region of the proline-rich domain or mutating the SH2 domain of Slp76 all resulted in defective cluster assembly, persistence and movement. The 3YF mutant assembled into very short-lived clusters whereas the other two mutants did not assemble into clusters at all, which indicated that these two domains are both essential for recruitment of Slp76. Furthermore GADS was unable to enter LAT-containing clusters in the absence of Slp76 and LAT clustering was not only dependent on the presence of SLP76 but was also dependent on its phosphorylation as the clustering of the 4YF LAT mutant failed to cluster. Collectively these results illustrated the importance of the LAT-GADS-SLP76 trimer (Bunnell et al., 2006). Although the microclusters of Slp76 and Zap70 quickly disappear from the central zone of the T-cell:APC contact interface, which is rich in TCR complex chains, microclusters of Zap70 and Slp76 persist at the periphery of the contact surface and these clusters are continuously generated (Yokosuka et al., 2005). These fluorescence

observations, mutagenesis and immunoprecipitation experiments clearly demonstrate the importance of Slp76 in TCR induced complex assembly.

Since J14 cells were successfully reconstituted with Slp76-RFP, studies were conducted to establish whether, like at the TCR, Slp76 clusters form in response to IFNAR ligation also. As a control, I also created another J14 cell line that I reconstituted with an empty RFP vector. In the same manner in which I created the Slp76-RFP J14 cell line, I selected cells for hygromycin resistance for two to three weeks until I gained a pool of cells stably expressing the RFP construct. However, there was no need to clone out individual cells in this case since the RFP was only intended to serve as a control and obviously, RFP is not expressed at a wild type level in Jurkat cells since it is not a native protein.

Cells were plated onto coverslips and stimulated with exogenous IFNα. Cells were then fixed, washed and mounted and visualised using confocal microscopy. Figure 4.7a shows that at the basal time point, Slp76 adopts a membrane localisation. Following stimulation, Slp76 moves into clusters at the cell surface, which can be seen at the 1 and 5 minute time points. By ten minutes Slp76 clusters are much larger and many have moved to a more nuclear localisation. By 30 minutes, the appearance of the cells is very similar to the basal time point and clusters have dissipated. The control J14 cell line expressing only RFP, display a diffuse localisation of RFP throughout the cells and this diffuse appearance does not change upon stimulation of the cells. This indicates that changes in localisation of the Slp76-RFP construct are not being hampered or influenced by the RFP tag.

Iimmunoflourescence was also used to verify whether changes in Slp76 localisation could be seen for wild type untagged protein in the Jurkat cell line (Figure 4.7b). Although there seemed to be quite a bit of background staining with the Cy3 tagged antibody, a membrane

localisation of Slp76 is distinguishable at early time points. Clusters of protein start to form by 5 minutes and similar to the J14 cells and by the ten minute time point these clusters are much more noticeable. These observations are in agreement with the localisation changes seen in reconstituted J14 cells.

In summary, upon IFNα stimulation, clusters of Slp76 form at the cell surface. This draws a parallel with observations of Slp76 localisation at the TCR since Slp76 is recruited into clusters at the cell surface. However, these clusters persist for a shorter time on the cell surface in response to TCR stimulation and move radially to perinuclear structures by 3 minutes (Bunnell et al., 2002).. In response to IFNAR, clusters also move to a more nuclear localisation, but this occurs at ten minutes and so takes longer to occur.

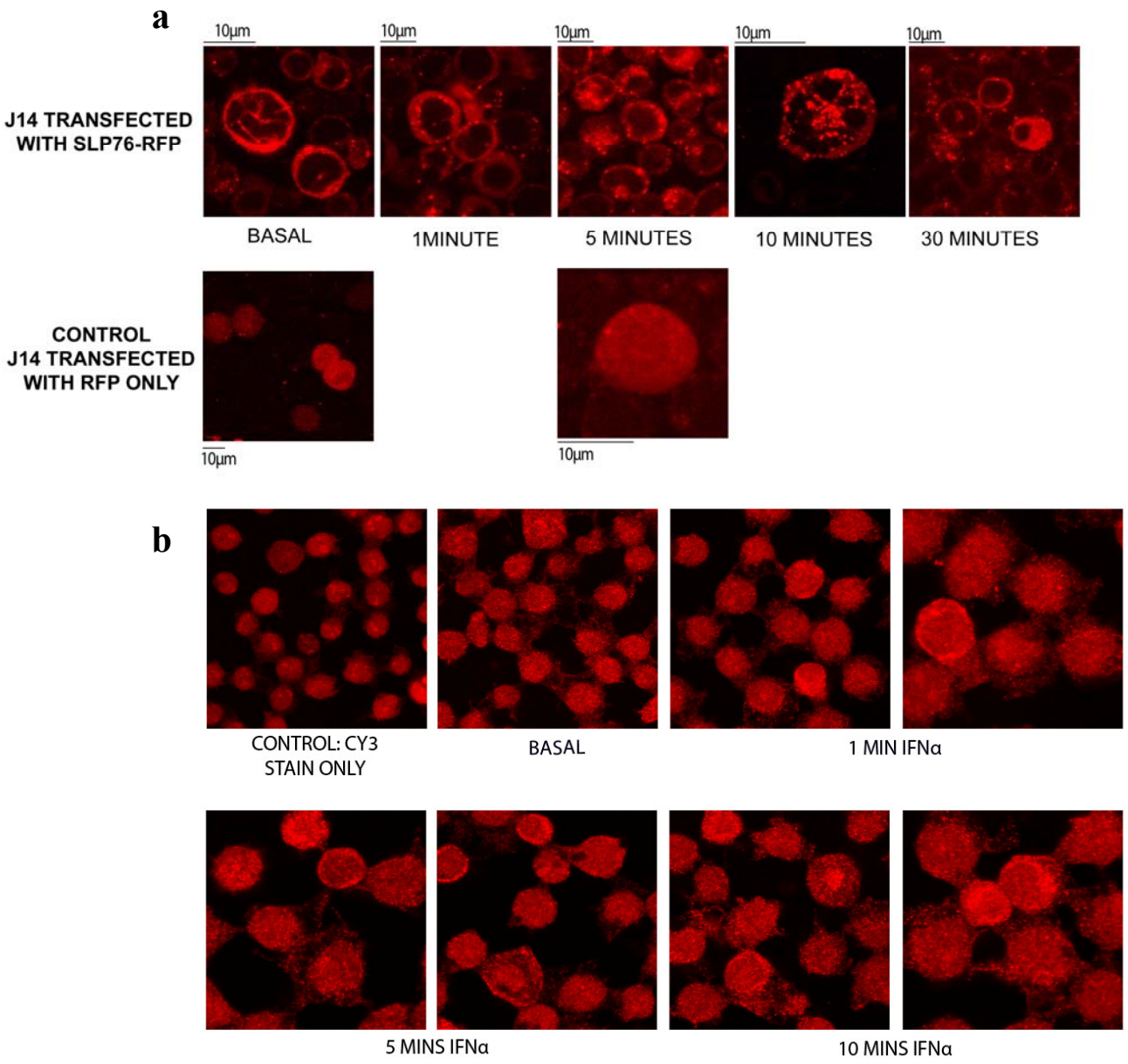


Figure 4.7 Slp76 forms clusters upon IFNα stimulation

(a) J14 cells stably reconstituted with Slp76-RFP (top panels) or, as a control, RFP only (bottom panels) were plated onto poly-L-lysine coated coverslips, starved for 2 hours and stimulated with 6000 U/ml Roferon for the time periods indicated. Cells were then fixed in 4% paraformaldeyde, washed and mounted and the distribution of Slp76-RFP was visualised using confocal microscopy.

(b) Jurkat cells were plated onto coverslip, serum starved for 2 hours and stimulated with 6000 U/ml Roferon for the time periods indicated. Cells shown in the upper left panel were stained with Cy3 only as control. The other panels show cells that were firstly treated with anti-Slp76 antibody. A secondary Cy3-conjugated antibody was then added. Cells were then visualised using confocal microscopy

4.4 Discussion

Although the role of Slp76 has been clearly defined at the TCR, its role at other receptors expressed upon the T-cell surface has been less well established. Slp76 is an adaptor protein and, at the TCR, helps nucleate protein-protein interactions within the inducible assembly of a macromolecular early signalling complex formed upon receptor stimulation. Slp76 is constitutively associated with GADs (Liu et al., 1999, Asada et al., 1999, Law et al., 1999, Dimasi, 2007, Seet et al., 2007) and upon TCR stimulation, the Slp76-GADs complex is recruited to the transmembrane adaptor, LAT, which is localised in glycolipid-enriched membrane domains (GEMs) resulting in a trimeric complex being formed (Boerth et al., 2000). Once recruited to lipid rafts, Slp76 is phosphorylated by Zap70 (Raab et al., 1997). Through its phosphotyrosines, Slp76 interacts with Vav, Nck, the p85 subunit of PI3K and Itk. Additionally Slp76 interacts with PLCγ, which also binds LAT. The SH2 domain of Slp76 is pivotal for binding ADAP and HPK-1 (interactions reviewed in Koretzky et al., 2006). Together, at the TCR, these multiple interactions within a stable multi-molecular complex that provides a platform for downstream signalling molecules to be recruited and activated, for example the exchange factor Ras, which commences the ERK MAPK cascade.

4.4.1 Slp76 is required at the TCR and IFNAR for ERK MAPK signalling

The importance of Slp76 in both T-cell development and T-cell signalling has been illustrated through the creation of Slp76 knockout mice and through the isolation of the J14 Slp76 deficient Jurkat T cell lymphoma cell line. Slp76 negative mice exhibit a block in the transition from double negative to double positive T-cells during thymic development and

hence display a complete absence of peripheral single positive (CD4+ or CD8+) mature T-cells. This block can not be overcome by treatment with anti-CD3 are can therefore be explained by the fact that Slp76 is requisite for pre-TCR signalling and defective signalling halts further T-cell development (Clements et al., 1998, Pivniouk et al., 1998). Creating reconstituted Slp76 deficent mice with an inducible Slp76 gene restores normal thymopoeisis when the gene is induced. In contrast inducible expression of Slp76 mutated at all three N-terminal tyrosines does not restore thymopoeisis, showing that Slp76 is required for and phosphorylated during pre-TCR signalling (Myung et al., 2001). Due to the fact that Slp76 knockout mice have no peripheral T cells, studies could not be performed to establish how absence of Slp76 affects signalling in mature Slp76 deficient T cells. The alternative was to use the J14 T cell lymphoma cell line too elucidate the role of Slp76 through comparison of signalling pathways with wild type Jurkat cells. It was found that, in response to stimulation through the TCR, these cells exhibited defects in ERK1/2 phosphorylation, NFAT transcription factor activation, IL-2 promoter activation, PLCγ phosphorylation and calcium flux (Yablonski et al., 1998). In agreement with these findings, my data also shows diminished ERK MAPK signalling in response to TCR stimulation and this reduction is almost equal to the diminished response observed in J.Vav1 cells (Figure 4.2a).

Previous studies have shown that the T-cell machinery is utilised at the IFNAR (Petricoin III et al., 1997, Lund et al., 1999, Ahmed et al., 2005). At the TCR, the sequential activation of CD45, Lck and Zap70 brings about Slp76 phosphorylation since Slp76 relies upon the kinase domain of tyrosine-phosphorylated Zap70 for phosphorylation (Raab et al., 1997). In addition, Slp76 and Vav directly associate, which requires an interaction between the SH2

domain of Vav and phosphotyrosines 112 and 128 within the acidic region of Slp76 and this interaction mediates downstream IL-2 transcription (Tuosto et al., 1996, Raab et al., 1997, Wu et al., 1996, Fang & Koretzky, 1999). My own studies show that Vav is necessary for IFNAR induced ERK1/2 MAPK signalling and previous studies show that the presence of both Lck and Zap70 are also absolutely necessary for this pathway (Lund et al., 1999, Ahmed et al., 2005). Therefore, it was predicted that Slp76 would also be involved in this pathway if events mimic those at the TCR.

As hypothesised, my results now add to the previous evidence of TCR machinery being utilised by the IFNAR for signalling since it is shown in this chapter for the first time that Slp76 is also required for downstream ERK1/2 MAPK signalling as this pathway is abolished in the absence of Slp76. The level of reduction is even more profound than when Vav is absent. Unlike Slp76, Vav is not an adaptor and does not solely act to mediate protein-protein interactions. Therefore, in the absence of Vav, some Grb2-SOS containing complexes may still be recruited to other adaptor domain-containing proteins localised at the IFNAR, but the absence of Slp76 may totally disrupt complex formation leading to total abrogation of ERK MAPK signalling. Restoring expression of Slp76 restores the response, which verifies that it is the absence of Slp76 responsible for impaired ERK phosphorylation rather than any other defect in J14 cells. Although it was found that Slp76 abolition perturbs ERK1/2 phosphorylation, the effect upon other IFNAR-induced signalling cascades was not explored. Since the primary signalling cascade activated at the IFNAR is the Jak/STAT pathway, further experiments are now required to clarify whether the absence of Slp76 impedes the activation of any proteins involved in this signalling module, for example, by comparing JAK and STAT phosphorylation levels between Jurkat and J14 cells.

4.4.2 Phosphorylation of Slp76 at the IFNAR

In response to TCR antigen recognition, Slp76 is phosphorylated upon three tyrosine residues within the N-terminal acidic region (Fang et al., 1996, Jordan et al., 2006). Previous studies have shown through immunoprecipitation that Slp76 is phosphorylated within 15 seconds of TCR stimulation and that phosphorylation is maximal at 1 minute. A band that corresponds to Vav is also seen at 1minute (Pacini et al., 2000).

My results show for the first time that Slp76 is also inducibly phosphorylated upon addition of IFNα. Similar to results at the TCR, phosphorylation can clearly be seen after one minute of IFNα stimulation and this persists for at least 10 minutes. In addition a band that corresponds to the size of Vav was visible. Reprobing with a Vav specific antibody showed that this band corresponded to Vav. This shows that Vav and Slp76 are both recruited into the same complex. The results of the previous chapter also suggest that a Vav-Slp76 interaction occurs at the IFNAR because immunoprecipitating with Vav results in a band of protein that corresponds to the size of Slp76 and, in the absence of Slp76, Vav is not phosphorylated suggesting that a physical interaction between the two proteins is mandatory for MAPK signalling. This is reminiscent of events at the TCR

In future, more detailed analysis of the interaction between the two proteins could be performed to see how the two proteins interact. It would be interesting to assess if Slp76 is still phosphorylated in Vav deficient J.Vav1 cells to give an indication of whether Vav is required upstream or downstream of Slp76. In addition, it would be interesting to ascertain whether a Vav-Slp76 interaction at the IFNAR requires the same regions of each to bind to one another as at the TCR i.e. whether the SH2 domain of Vav binds to phosphotyrosines 112 and 128 of Slp76. This could be assessed using the stable J14 mutant cell lines that I

created by cloning out the cells so that they express an equal level of the mutant constructs to the wild type level of Slp76 in Jurkat cells. Immunoprecipitations of IFNα stimulated lysates could then performed in each mutant cell line with either an anti-Vav or anti-Slp76 antibody, followed by Western blotting with an anti-phosphotyrosine antibody, to see whether individual mutations disrupt the interaction. Additionally the fluorescently tagged constructs that were created could be used to see if, firstly, the two proteins co-localise over time when IFNα is added and, secondly, whether the two proteins physically interact by performing Fluorescence Resonance Energy Transfer (FRET) experiments. J14 cells stably expressing RFP tagged Slp76 could be co-transfected with the eGFP tagged Vav construct described in chapter one in order to achieve these two facets. Immunoprecipitation experiments give information about whether two proteins of interest are found within the same complex, but are not necessarily representative of a physical interaction whereas FRET is a more precise way to assess whether two proteins physically interact as, for energy transfer to occur between the two flourophores of the tagged proteins of interest, proteins must be in a very close proximity of less than 9nm.

As mentioned in the previous chapter, T cells express three different isoforms of Vav: Vav1, Vav2 and Vav3. The most important for TCR signalling is Vav1 and Vav2 seems to play a minimal role (Fujikawa et al., 2003). However there is evidence that Vav3 is recruited at the TCR and this is dependent upon an interaction of the Vav SH2 domain with Slp76. In Lck, Zap70 or LAT deficient cells, binding of Vav3 to Slp76 no longer occured (Charvet et al., 2005). It would therefore, also be useful to establish whether Vav3 also binds Slp76 at the IFNAR and whether this also requires similar proteins to be present.

At the TCR, several reports gauged the contribution of individual tyrosine residues towards downstream ERK1/2 MAPK signalling by creating J14 cell lines reconstituted with single, double and triple Slp76 mutants (Fang et al., 1996, Jordan et al., 2006). In a similar fashion, I also created the same mutants in order to determine whether the downstream ERK MAPK pathway was directly dependent upon phosphorylation of Slp76. Reconstitution of J14 cells with the 3YF triple tyrosine mutant did not restore ERK1/2 phosphorylation, which shows that the interactions formed between these phosphotyrosines are necessary to stabilise a multi-molecular complex, which is imperative for downstream signalling events. Without phosphorylation, correct complex assembly would be abrogated. Preliminary data suggests that all three tyrosine residues are required in order for MAPK signalling to be restored since J14 cells reconstituted with any Slp76 mutant constructs did not display elevated MAPK signalling upon 5 minutes IFNα administration.

At the TCR, the phosphorylation of these tyrosine residues relies upon the presence of activated Zap70 (Raab et al., 1997). It would be beneficial, in future, to ascertain whether the phosphorylation of Slp76 at the IFNAR is also Zap70-dependent. To achieve this Jurkat and P116 cells that lack Zap70 expression could be stimulated over time with IFNα. An immunoprecipitation could then be performed using an anti-Slp76 antibody followed by Western blotting with anti-phosphotyrosine. It would be predicted that, in the absence of Zap70, Slp76 tyrosine phosphorylation would be abolished since evidence so far suggests that Slp76 interactions at the IFNAR proceed in the same manner as at the TCR.

The three N-terminal tyrosine residues have been found to mediate interactions with not only Vav, through tyrosines 112 and 128, but these two residues also interact with the Nck adaptor protein (Wunderlich et al., 1999) and the p85 subunit of PI3K (Shim et al., 2004).

Phosphotyrosine 145 mediates the interactions with Itk, a Tec family kinase protein that mediates cytoskeletal reorganisations (Su et al., 1999). It would be interesting to determine whether, at the IFNAR, these residues interact with any of these binding partners. Alternatively they may interact with IFNAR specific proteins. In addition to the N-terminal tyrosine residues within the acidic region, the other domains are also responsible for mediating a number of important protein-protein interactions at the TCR. A core RxxK motif located within the proline-rich region of Slp76 is responsible for the interaction with SH3 domain of GADs and this interaction occurs with very high affinity and specificity, as shown through crystal structure studies, and is required for downstream NFAT activation (Lui et al. 1999, Dimasi, 2007, Seet et al., 2007). The proline-rich region also binds to PLCγ (Yablonski et al., 2001). A small region distal to the acidic region at the boundary of the proline-rich region has been reported to bind Lck (Sanzenbacher, 1999). The SH2 domain binds to phosphorylated ADAP (SKAP-130/Fyb) and HPK-1, which is a serine-threonine kinase (Musci, 1997b, da Silva, 1997, Sauer et al., 2001). Interactions by the proline-rich regions have also been found to be required for T cell development. For instance, mice bearing a deletion in the GADs interacting region of Slp76 extending from amino acids 224-244 of the proline-rich region were defective in thymopoiesis.. However, in mice that expressed Slp76 bearing a point mutation in the SH2 region (R448K), thymopoiesis was rescued and the peripheral T cells only had defects in mature T-cell signalling, illustrating that the SH2 domain interactions are not mandatory during T-cell development (Myung et al., 2001).

Further experiments could involve the creation of Slp76 constructs bearing point mutations, truncations or deletions in other domains of the protein, which could then be transfected back into J14 cells at a wild-type expression level in order to determine the level of requirement of

the other domains for downstream signalling in response to IFNα. It would be particularly interesting to study the effect of mutating the GADs binding region since these two proteins are constitutively associated (Liu et al., 1999, Asada et al., 1999, Law et al., 1999, Dimasi, 2007, Seet et al., 2007). Upon TCR stimulation, the Slp76-GADs complex is recruited to the transmembrane adaptor, LAT, which is localised in glycolipid-enriched membrane domains (GEMs) resulting in a trimeric complex being formed (Boerth et al., 2000). It is possible similar events take place at the IFNAR.

4.4.3 Slp76 localisation upon IFNAR stimulation

Further evidence that Slp76 is important for IFNAR signalling is provided from the fluorescence localisation experiments. Slp76 migrates from a uniform membrane localisation to clusters upon IFNα stimulation. At the TCR extensive studies have been conducted to establish how the localisation of Slp76 varies over time. It was found that Slp76 forms clusters at the cell surface within 15 seconds, which also contain the TCRβ subunit, TCRζ subunit, Zap70, Lck, Grb2, GADs, Cbl, PLCγ and LAT (Bunnell et al., 2002, Yokosuka et al., 2005, Braiman et al., 2006, Bunnell et al., 2006). The clusters of Slp76 behave uniquely when contrasted with the other TCR signalling molecules studied so far because the Slp76-rich clusters translocate radially within 2-3 minutes of being plated onto a ligand-coated coverslip to peri-nuclear structures (Bunnell et al, 2002). In the absence of Zap70, Lck or LAT, Slp76 clustering is compromised and any clusters that do form do not translocate radially nor do they perist at the cell surface (Bunnell et al., 2006). This highlights the necessity for Slp76 to form key interactions at the TCR within a complex and absence of any component disrupts normal behaviour.

In parallel to observations upon TCR engagement, my results show that Slp76 also forms clusters upon the addition of IFNα and these too appear to move laterally towards the nucleus by 10 minutes. It would be useful to implement more sensitive techniques to track the movement of individual clusters in the same manner as experiments performed at the TCR. Also co-transfecting Slp76-RFP containing J14 cells with other fluorescently tagged proteins would allow any co-localisation to be viewed e.g. between Zap70 and Slp76 or between Vav and Slp76.

Additionally, it would also be interesting to determine how the mutant constructs I created behave when cells are stimulated with IFNα in order to determine which, if any, tyrosine residues are required for recruitment. At the TCR, it was found that a 3YF mutant of Slp76 migrated into clusters, but these dissipate much faster than wild-type Slp76 clusters (Bunnell et al., 2006). It is possible that similar behaviour is observed at the IFNAR.

Slp76 plays an important role at the TCR in orchestrating cytoskeletal changes. Two important interactions formed that mediate theses cytoskeletal rearrangements are those formed with Vav and Nck. Vav is recruited to a complex consisting of Slp76, Nck and two other binding partners of Nck, PAK1 and WASP. Vav, Nck, PAK1 and WASP then mediate actin polymerisation (Ku et al., 2001, Zeng et al., 2003). Another protein that binds the SH2 domain of Slp76 named ADAP also mediates cytokskeletal changes as it is be required for 'inside-out' upregulation of the LFA-1 and VLA-4 integrins also (Koretzky, 2004). Further explorations are required to find out whether IFNα also induces cytoskeletal changes.

4.4.4 General Conclusions

In summary, the results of this chapter show for the first time that the presence of Slp76 in Jurkat cells is required for downstream ERK1/2 MAPK signalling in response to IFNα. This requirement is phosphorylation dependent since reconstitution of the J14 cell line with a Slp76 construct bearing mutations in all three N-terminal tyrosine residues does not result in the restoration of ERK MAPK signalling. Immunoprecipitation experiments also verify that Slp76 is phosphorylated upon IFNα stimulation. Furthermore, Slp76 clusters appear upon IFNα stimulation suggesting that it may be recruited to lipid rafts. These findings are all strikingly similar to the role of Slp76 at the TCR. Further experiments are now necessary in order to examine more precisely whether Slp76 is phosphorylated in the same fashion as at the TCR and whether it is recruited to the cell surface in a comparable manner. Also, further binding partners of Slp76 at the IFNAR require characterisation. These observations will help to further elucidate the cross talk between the TCR and IFNAR. In addition to this new found role at the IFNAR, Slp76 is also required at several other receptors such as the CXCR4 receptor, which binds to the ligand SDF-1α and induces prolonged ERK1/2 phosphorylation. The absence of Slp76 in the J14 cell line disrupts downstream ERK MAPK as a result of SDF-1α stimulation when compared with wild type Jurkat cells. When J14 cells are reconstituted with Slp76, the response is restored, but when the cells are reconstituted with a 3YF Slp76 mutant, the response is not restored (Kremer et al., 2003). This is similar to my own observations at the IFNAR and shows that Slp76 is utilised not just by the TCR antigen receptor, but also by both chemokine and cytokine receptors expressed upon the T-cell.

CHAPTER 5

Investigating cross-talk between the TCR and IFNAR

5.1 Introduction

A body of evidence has accumulated that illustrates that the IFNAR and TCR recruit an overlapping subset of proteins in order for non-STAT signalling pathways such as the ERK MAPK pathway to proceed. Previous studies have illustrated the necessity for Zap70, Lck and CD45 in mediating the antiviral effects of IFNα (Petricoin III et al., 1997., Lund et al., 1999, Ahmed et al., 2005). It was also revealed by Petricoin III et al. (1997) that CD45, Lck and Zap70 follow analogous sequential activation patterns to that seen at the TCR. Therefore a model was proposed by the authors of the paper whereby CD45 associates with the IFNAR upon IFNα ligation, which subsequently recruits and dephosphorylates Lck, which is then conferred with the ability to phosphorylate, and hence activate, its downstream target Zap70. The previous two chapters show that both Vav and Slp76 are phosphorylated in T cells in response to IFNα and that both proteins are also mandatory for the ERK MAPK cascade brought about by this receptor. Furthermore, like their counterparts (CD45, Zap70 and Lck), the modes in which they are phosphorylated replicates that described at the TCR. As there seem to be many analogous signalling events at both the TCR and IFNAR, which command an overlapping subset of proteins, it is probable that crosstalk occurs between the two receptors at some level.

5.2 Aims

In this chapter, investigations were carried out to establish whether crosstalk occurs between the TCR and IFNAR by determining if the IFNAR requires a functional TCR to be expressed upon the cell surface in order for signalling through the ERK MAPK pathway to occur. There are two possibilities of how the IFNAR utilises TCR-associated proteins for

signalling. The first possibility is that the IFNAR and TCR signal independently of one another and therefore proteins are recruited to either the TCR or IFNAR early signalling complex separately upon stimulation (Figure 5.1a). The second possibility is that the IFNAR requires the TCR to be expressed in order to allow TCR associated proteins to be recruited into a complex. Therefore the TCR may act as a scaffold and may co-localise with the IFNAR, allowing the correct proteins to be recruited so that the ERK MAPK pathway is triggered (Figure 5.1b). If the TCR was required for signalling events through the IFNAR, this would indicate that crosstalk occurs between the IFNAR and TCR

In order to verify whether crosstalk does occur between the IFNAR and TCR at the receptor level, firstly, whether a fully assembled TCR was necessary for ERK MAPK phosphorylation upon IFNα stimulation and, secondly, whether full phosphorylation of T-cell associated proteins such as Slp76 and Vav still occurs in the absence of the TCR were investigated. Finally, the phosphorylation state of the TCR ζ subunit was observed in order to assess whether the TCR ζ chains became tyrosine phosphorylated upon stimulation of the IFNAR.

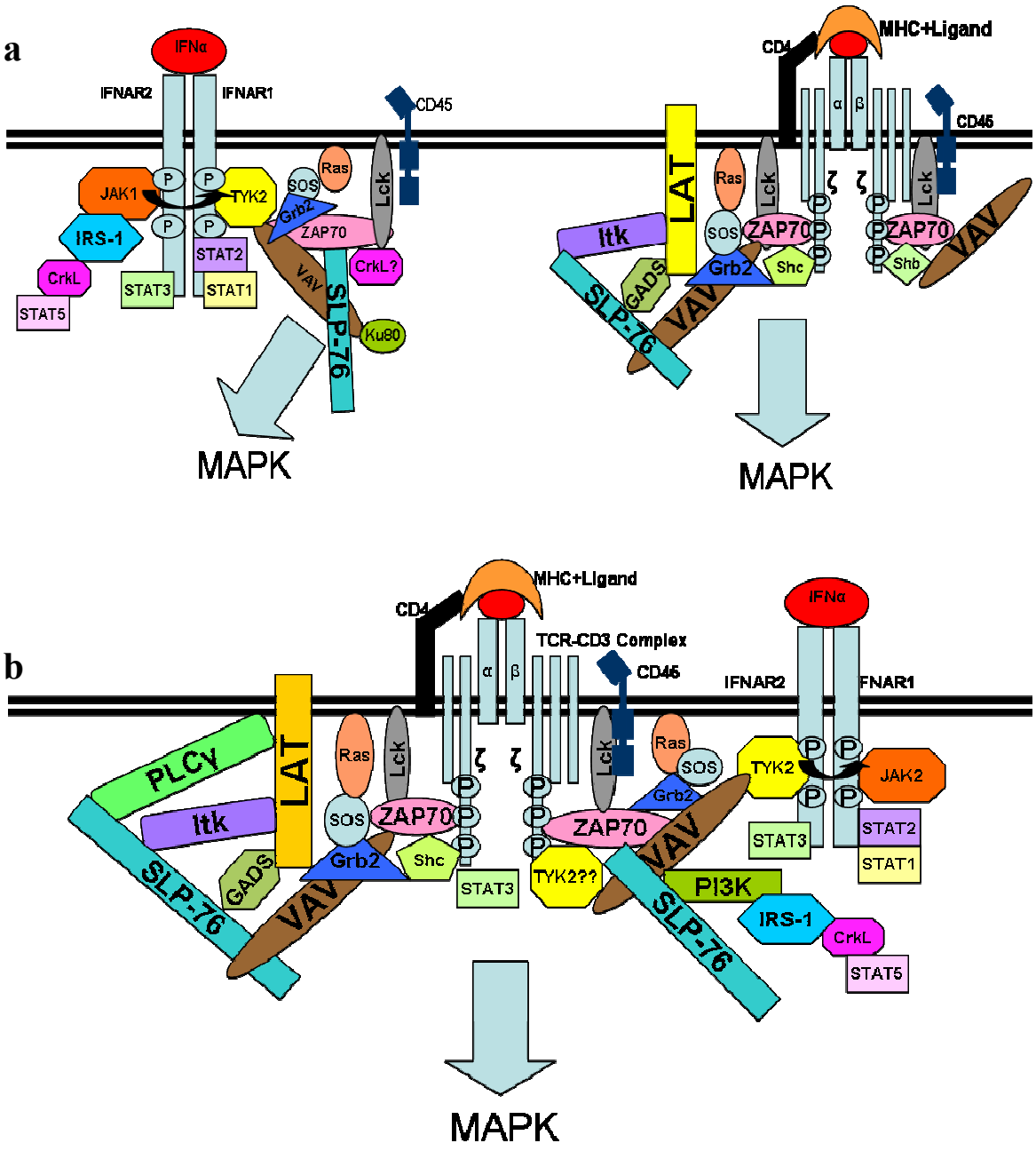


Figure 5.1 Diagrammatic representation of two possibilities of how the IFNAR recruits TCR-associated proteins into a complex.

(a) No Cross-talk occurs between the TCR and IFNAR: T cell receptor proteins are recruited to the TCR or IFNAR independently of one another.

(b) Cross-talk occurs between the TCR and IFNAR: Recruitment of the TCR machinery to the IFNAR requires a functional TCR to be present so that complex assembly can occur.

5.3 Results

5.3.1 Utilising TCR deficient cells to demonstrate the requirement for the TCR receptor for the ERK1/2 response following IFNAR ligation

In order to determine whether the TCR itself was required for IFNα-induced ERK MAPK signalling, the JRT3-T3.5 was acquired (which was a generous gift from A. Weiss). The JRT3-T3.5 cell line is deficient in the β-subunit of the TCR and consequently proper assembly of a complete, functional TCR can not occur. The complete ensemble of the TCR α and β chain with the CD3 complex subunits normally occurs in endosomal compartments before the complete TCR is transported to the T-cell surface. The absence of any of its subunits results in no expression of the TCR on the cell surface as this transportation step does not occur (Weiss & Stobo, 1985). Therefore, the JRT3-T3.5 cell line would allow determination of whether the absence of a functional TCR on the cell surface would impinge upon the ERK MAPK response emanating from the IFNAR. The PF2.4 cell line was also acquired. These are cells in which the β-subunit has been stably reconstituted back into the JRT3-T3.5 cell line using an expression vector and consequently a functional, fully assembled TCR is once more expressed upon the T-cell surface (Ohashi et al., 1985). This would allow verification of whether any defects observed in the JRT3-T3.5 cell line could be restored in the reconstituted cell line thereby indicating that the absence of the TCRβ chain is directly responsible for defects in signalling rather than the cells being unresponsive for another reason.

5.3.2 Expression levels of T-cell proteins are equivalent between the cell lines

In order to ensure that the JRT3-T3.5 cells only lacked expression of the TCRβ chain and not other components of the TCR signalling machinery, equal quantities of lysates from Jurkat cells and JRT3-T3.5 cells were used for a western blot, which was then probed with Slp76 and Vav antibodies (Figures 5.1b and 5.1d respectively) as these are key proteins involved in TCR signalling and IFNAR signalling. Lysates from both J14 and J.Vav1 cell lines were run on the same gel as a further control. The same lysates were used for a Western blots with anti-TCR ζ and anti-IFNAR1 antibodies to ensure that both of these receptor subunits were expressed at equal levels in all cell lines (Figures 5.2a and 5.2c). This was to ensure that any noticeable signalling impediments in any of the knockout cell lines were not a consequence of a lack of or reduced levels of receptor. Expression of the proteins probed for was equal in all cell lines and so this illustrated that these cell lines did offer useful and reliable models for further experimentation.

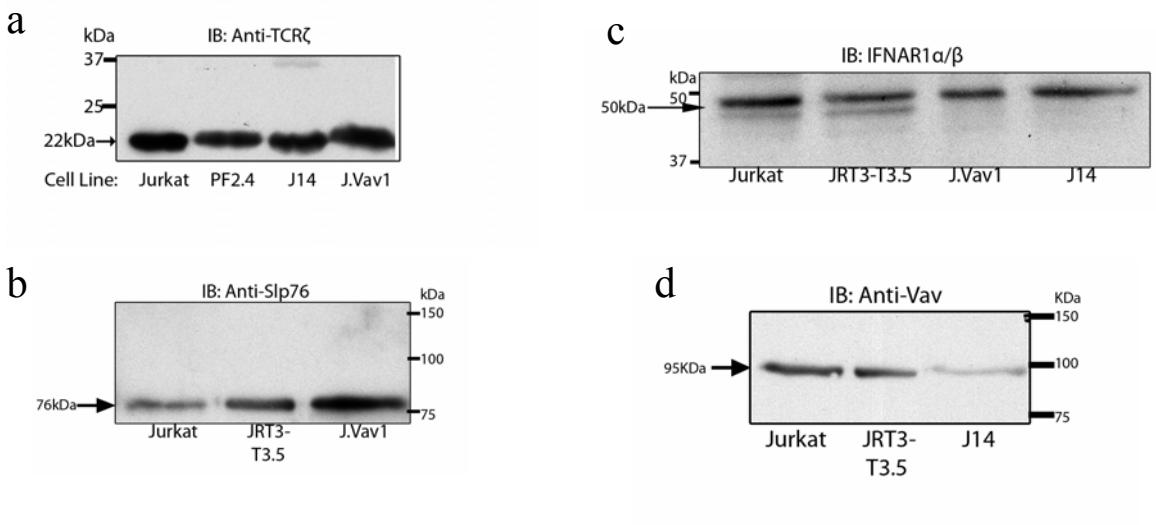


Figure 5.2 Expression of proteins between all cell lines is equal

(a), (b), (c) and (d) Equal amounts of lysates from the cell lines indicated were resolved on an SDS-PAGE gel and western blotted with the antibodies indicated

5.3.3 JRT3-T3.5 cells are unresponsive to OKT3, which is indicative of a lack of expression of the functional TCR complex at the cell surface

If a functional TCR is not expressed upon the cell surface of the JRT3-T3.5 cell line, this would render the cells unresponsive to TCR ligands and therefore no effects upon downstream signalling would be predicted to occur following addition of ligand. Upon reconstitution of the TCRβ deficient cells, the supposition would be that the responses are restored upon TCR ligation. In order to check that this is indeed the case, Jurkat, JRT3-T3.5 and PF2.4 cell lines were all stimulated through the TCR with the OKT3 antibody under similar conditions over a time course of 30 minutes. Phosphorylated ERK1/2 was then probed for as numerous studies conducted using Jurkat cells, including that which are presented in this thesis, have shown an upregulation in the ERK MAPK pathway following TCR ligation. This is essential for cell divisions and growth during an adaptive immune response. The results demonstrate that ERK1/2 phosphorylation is induced 5 minutes after OKT3 addition in both the Jurkat and PF2.4 cell line and this elevated level of phospho-ERK1/2 still persists at 30 minutes (Figure 5.3a). In contrast, the TCRβ deficient JRT3-T3.5 cells display no increase in ERK1/2 phosphorylation at all during the time course and the level of phospho-ERK1/2 remains at basal levels. To ensure that each lane contained the same amount of protein, the blot was then reprobed with total ERK1/2, which confirmed that the results were an accurate representation and not just a by-product of incorrect loading. The graphical representation of the intensity of the phospho-ERK1/2 bands shows even more clearly the time-dependent heightened response to OKT3 of the Jurkat and PF2.4 cell lines are similar whereas the graphical line representing the JRT3-

T3.5 phosphorylation level data remains at a basal level (Figure 5.3b). This undoubtedly shows that, since the JRT3-T3.5 cells lack the TCRβ subunit, this TCR-dependent ERK MAPK signalling no longer occurs. The results also demonstrate that the PF2.4 cells function normally and are able to induce an ERK response comparable to parental Jurkat cells.

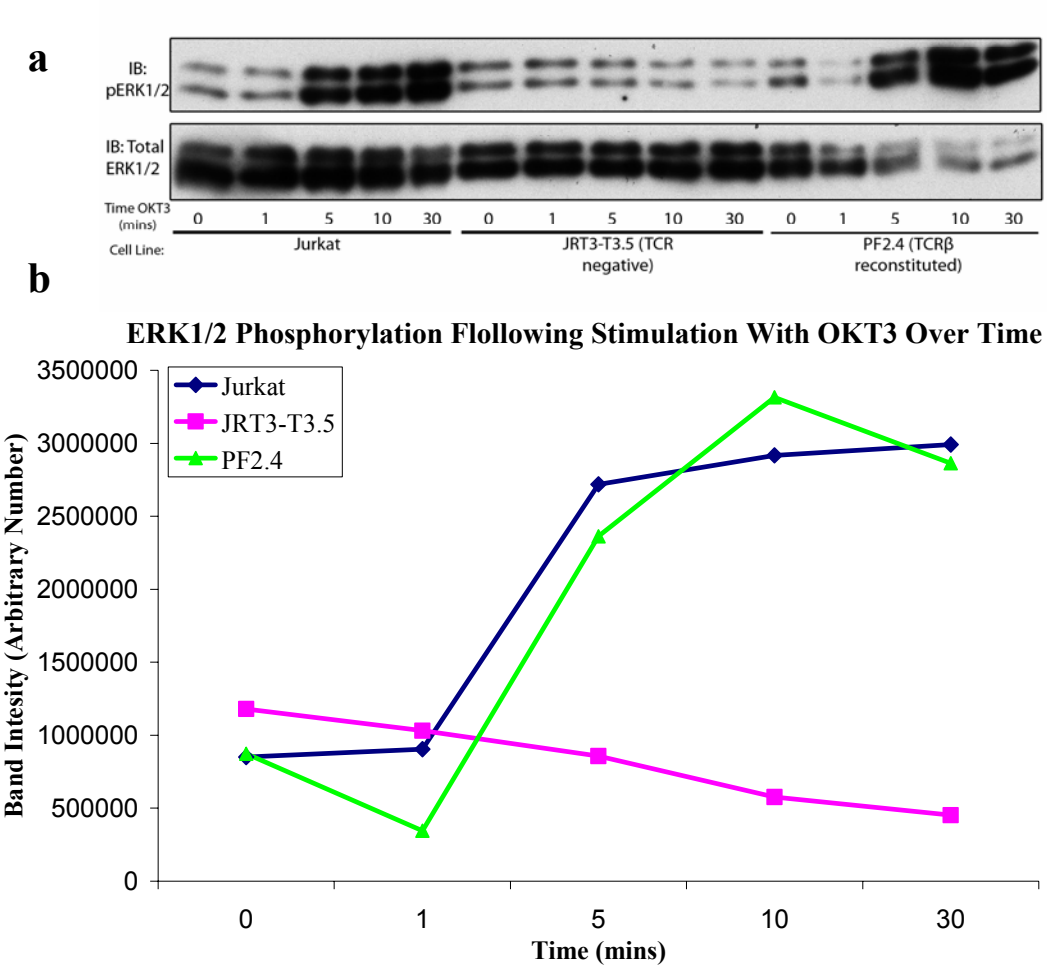


Figure 5.3 TCRβ deficient JRT3-T3.5 cells do not respond to OKT3

(a) Jurkat, JRT3-T3.5 and PF2.4 cells were stimulated with 1 μg/ml OKT3 for the time course indicated. Cells were then lysed and equal quantities of were resolved through SDS-PAGE and then western blotted with anti-phosphoERK1/2 (upper panel). The membrane was then striped and reprobed with anti-total ERK1/2 to ensure equal loading (lower panel)

(b) The phospho-ERK1/2 bands were then quantified using densitometry analysis and the data were plotted graphically.

5.3.4 The TCR is required for ERK MAPK phosphorylation in response to IFNα

Having established that the TCRβ chain is required for the TCR-induced ERK MAPK cascade, the next step was to discover whether the absence of the TCR would also affect the IFNα-induced ERK MAPK response. In a comparable fashion to the stimulation carried out with OKT3, Roferon was used to stimulate Jurkat, JRT3-T3.5 and PF2.4 cells over a time course of 30 minutes under the same conditions. Equal amounts of lysates were Western blotted for phosphorylated ERK1/2 and reprobed with total ERK1/2 to ensure equal loading (Figure 5.4a). The results show that in the TCRβ-deficient JRT3-T3.5 cell lysates, ERK1/2 phosphorylation is barely detectable over the time course. Conversely, in Jurkat cells the levels of ERK phosphorylation are clearly raised within a minute, falling back to basal levels by 30 minutes, which is in agreement with the previously reported observations of ERK MAPK in Jurkat cells (Ahmed et al., 2005). In the reconstituted PF2.4 cells, ERK phosphorylation is also induced upon IFNAR ligation and largely mimics that of Jurkat cells. This indicates that the lack of ERK1/2 phosphorylation is directly linked to the absence of the TCRβ chain as ERK1/2 phoshorylation can be restored upon re-expression of the TCRβ chain. The phospho-ERK1/2 bands were also quantified and represented graphically and this patently shows a clear difference over time of ERK phosphorylation levels between cells with and without a functional TCRβ chain (Figure5.4b).

In addition to down regulation of ERK1/2 activation, IFNα stimulation of cells lacking the intact TCR also failed to generate a MEK1/2 response suggesting the defect is this cascade occurs upstream of ERK1/2 since MEK is the serine/threonine kinase that phosphorylates ERK (Figure 5.4c). It is possible that the entire MAPK cascade is defective. This would be

most likely due to the fact that the absence of the TCR would prevent the correct assembly of proteins at the cell membrane thereby halting the correct compartmentalisation of proteins within lipid rafts, which could abrogate correct recruitment of proteins required to instigate MAPK signalling and/or prevent them from accessing their substrates.

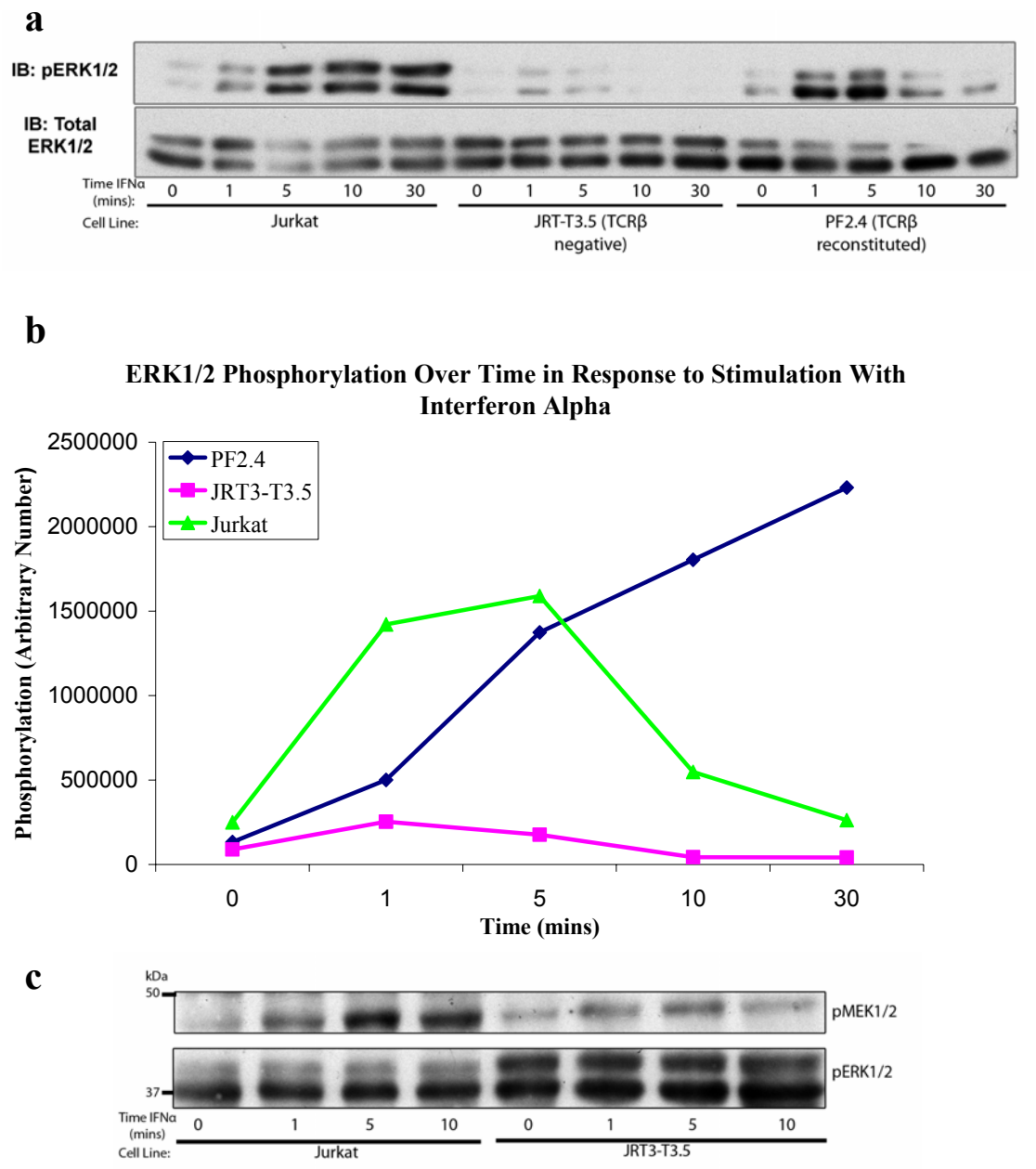


Figure 5.4 A functional TCR is required in order for the IFNAR to signal through the ERK MAPK pathway

(a) Jurkat, JRT3-T3.5 and PF2.4 cells were stimulated over the time course shown with Roferon. Cells were then lysed and resolved through SDS-PAGE followed by western blot analysis with anti-phospho-ERK1/2 (upper panel). The membrane was then stripped and reprobed with a total anti-ERK1/2 antibody. **(b)** The bands of phospho-ERK1/2 were quantified using densitometry and the data gained was plotted on a graph.

(c) Jurkat and JRT3-T3.5 cells were stimulated with 6000 U/ml Roferon over time and lysed. Lysates were then used for a western blot with anti-phospho-MEK1/2 (upper panel). The membrane was then stripped and reprobed with anti-ERK1/2 (lower panel). (a) and (c) are representative of blots from three individual experiments

5.3.5 Tyrosine phosphorylation of proteins differs between Jurkat cells and JRT3-T3.5 cells over time

Hereafter, whether the lack of the TCR accounted for any global defects in tyrosine phosphorylation was explored. IFNα stimulated lysates were obtained from Jurkat cells and JRT3-T3.5 cells, stimulated over time, and probed for anti-phosphotyrosine, allowing all tyrosine phosphorylated proteins to be seen (Figure 5.5a). Levels of phosphorylation were generally higher in Jurkat cells, even at basal levels, when compared with JRT3-T3.5 cells and this is most probably accounted for by low tonic TCR signalling in the absence of ligand. By 5 minutes, phosphorylation reached a maximum in both cell lines albeit there was a slightly wider spectrum of phosphorylated protein bands apparent in Jurkat cells. There were a selection of bands present in Jurkat cells that were not detectable in the JRT3-T3.5 cell line at all such as a band just above 75kDa and also a band around 70kDa. These bands may correspond to Slp76 and Zap70. There was also another prominent band of protein at around 150kDa, which was not phosphoryated in JRT3-T3.5 cells. These data imply that levels of phosphorylation are lower in the absence of the TCR and also suggest that some proteins can not be phosphorylated in the absence of the TCR.

To determine if the TCR is required as a scaffold for complex assembly in response to IFNAR ligation, differences were looked for in the proteins immunoprecipitated in the presence or absence of the TCR. Firstly, an immunoprecipitation was performed with an anti-vav antibody. The level of Vav phosphorylation appeared slightly lower in the JRT3-T3.5 cells than in Jurkat cells. In JRT3-T3.5 cells there Vav phosphorylation did increase slightly in JRT3-T3.5 but only at 5 and 10 minutes whereas in Jurkat cells Vav

phosphorylation occurred much faster. (Figure 5.5b shows Vav phosphorylation only and Figure 5.5c shows the whole blot). Secondly, it was assessed whether the magnitude of phosphorylation of Slp76 was equivalent in both Jurkat and JRT3-T3.5 cells by immunoprecipitating IFNα-stimulated lysates from both cell lines with an anti-Slp76 antibody followed by immunoblotting with an anti-phosphotyrosine antibody. Slp76 phosphorylation was higher in Jurkat cells when compared with the JRT3-T3.5 cells. In addition, bands were visible in Jurkat cells that were absent in JRT3-T3.5 cells such as a band at around 95kDa, which may correspond to Vav as this is a widely acknowledged binding partner of Slp76 (Figure 5.5d). These blots indicate that Slp76 and Vav can not be maximally phosphorylated when the TCR is not expressed. Had time allowed, further investigations into differences in protein phosphorylation in Jurkat and JRT3-T3.5 cells would have been carried out involving further sets of immunoprecipitations with other antibodies.

Figure 5.5 (OVERLEAF): In the absence of the TCR, some proteins are phosphorylated at a lower level.

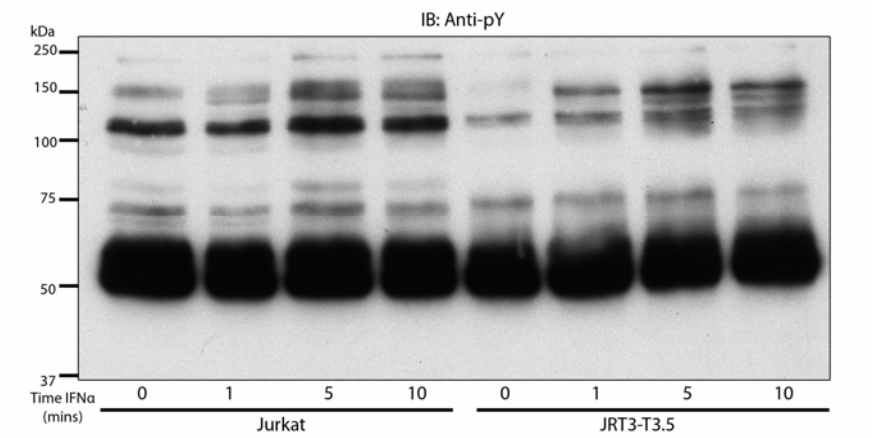
(a) Cell lysates from Roferon stimulated Jurkat and JRT3-T3.5 cells were resolved via SDS-PAGE and western blotted with a total anti-phosphotyrosine antibody

(b), (c) and **(d)** Jurkat and JRT3-T3.5 cells were stimulated with 6000 U/ml Roferon over time and lysed. Cell lysates were then immunoprecipitated with the antibodies indicated and then resolved through SDS-PAGE and Western blotted with anti-phosphotyrosine. Membranes were then stripped and reprobed with total Vav as shown in the lower panels.

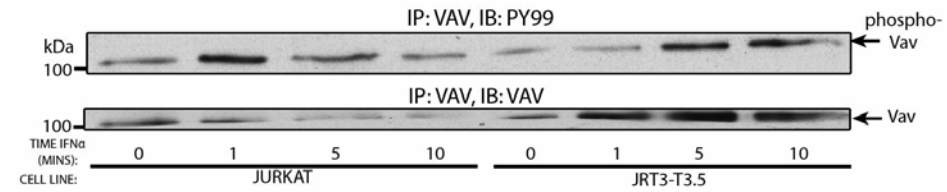
(b) and (c) are pictures of the same blot. (b) shows the level of phospho-Vav and total Vav only and was exposed for a shorter period of time whereas (c) is a longer exposure of the blot and shows the entire blot.

All blots are representative of three independent experiments.

a


IB: Anti-pY
kDa
250
150
100
75
50
37
Time IFNα (mins)
0 1 5 10 0 1 5 10
Jurkat
JRT3-T3.5

b


IP: VAV, IB: PY99
phospho-Vav
kDa
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IP: VAV, IB: VAV
Vav
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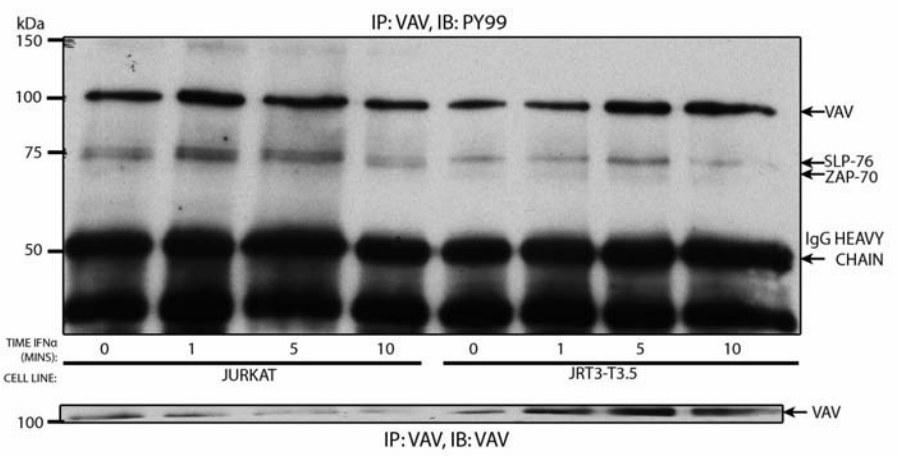
TIME IFNα (MINS):
0 1 5 10 0 1 5 10
CELL LINE:
JURKAT
JRT3-T3.5

c


IP: VAV, IB: PY99
kDa
150
100
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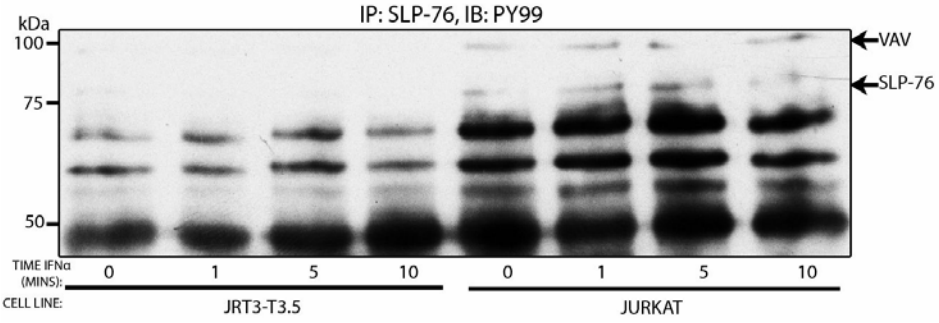
VAV
SLP-76
ZAP-70
IgG HEAVY CHAIN
TIME IFNα (MINS):
0 1 5 10 0 1 5 10
CELL LINE:
JURKAT
JRT3-T3.5
100
VAV
IP: VAV, IB: VAV

d


IP: SLP-76, IB: PY99
kDa
100
75
50
VAV
SLP-76
TIME IFNα (MINS):
0 1 5 10 0 1 5 10
CELL LINE:
JRT3-T3.5
JURKAT

5.3.6 Is the TCR is phosphorylated in response to IFNAR ligation?

As mentioned, a well documented example of crosstalk occurs between the TCR and the CXCR4 chemokine receptor. One way in which TCR-CXCR4 receptor crosstalk has been analysed, is through detection of the phosphorylation state of the ζ chains of the TCR-associated CD3 complex (Kumar et al., 2006, Patrussi et al., 2007). Similarly, it was examined whether the IFNAR could induce separate signalling events at the TCR, resulting in phosphorylation of ITAMs within the intracellular domains of the chains. This could potentially lead to the recruitment of proteins such as Zap70, Vav and Slp76, which are all constituents of proximal signalling events arising from IFNα stimulation.

The TCR ζ chains comprise the lion's share of the phosphorylation sites within the TCR complex. Out of the ten ITAM motifs encompassed within the TCR chains, the two ζ chains possess six, three per chain. Upon TCR ligation, it is these two chains that are phosphorylated first and so they are involved at the earliest level of TCR signalling. It was logical therefore, to determine whether these two chains are phosphorylated in upon IFNAR activation, since these studies that the TCR is a requirement for the ERK MAPK cascade generated by IFNα. To accomplish this feat, Jurkat cells were stimulated over a short time course with IFNα. Cells were lysed and immunopreceipitated with an anti-TCRζ antibody, following by Western blot analysis with an anti-phospho-tyrosine antibody to verify whether the chain had been phosphorylated or not (Figure 5.6a). Despite many repetitions of this experiment, utilising both monoclonal and polyclonal antibodies, conclusive evidence of ζ chain phosphorylation was not forthcoming and there were few differences between unstimulated cells and stimulated cells. Two blots only are shown in Figures 5.6a and 5.6b, which are representative of the many repetitions. It is possible that

IFNAR utilises constitutively tyrosine phosphorylated, rather than inducibly tyrosine phosphorylated TCR subunits. This is in accordance with findings arising from the exploration of ζ chain phosphorylation upon SDF1α stimulation, the ligand of the CXCR4 receptor. Despite many TCR signalling proteins being ascribed a role at the CXCR4 receptor in their report and despite the finding that ERK MAPK signalling was similarly impaired in TCRβ deficient Jurkat cells upon SDF-1α administration, no difference in TCRζ phosphorylation was observed (Kumar et al., 2006). However other results using a western blot only approach rather than immunoprecipitation, did suggest ζ chain phosphorylation when the CXCR4 receptor was stimulated (Patrussi et al., 2007). This study utilised a phospho-TCRζ antibody so it would have been interesting to see if, through a western blot only approach, phosphorylation of the ζ chain was observed. Despite having failed to visualise any phosphorylation changes at the TCR, when IFNα stimulated lysates were probed with a TCRζ antibody, it was found that the level of soluble receptor decreased even though equal amounts of lysates were loaded (Figure 5.6b). This suggests that perhaps the ζ chain is downregulated as the amount of receptor was approximately half that of basal.

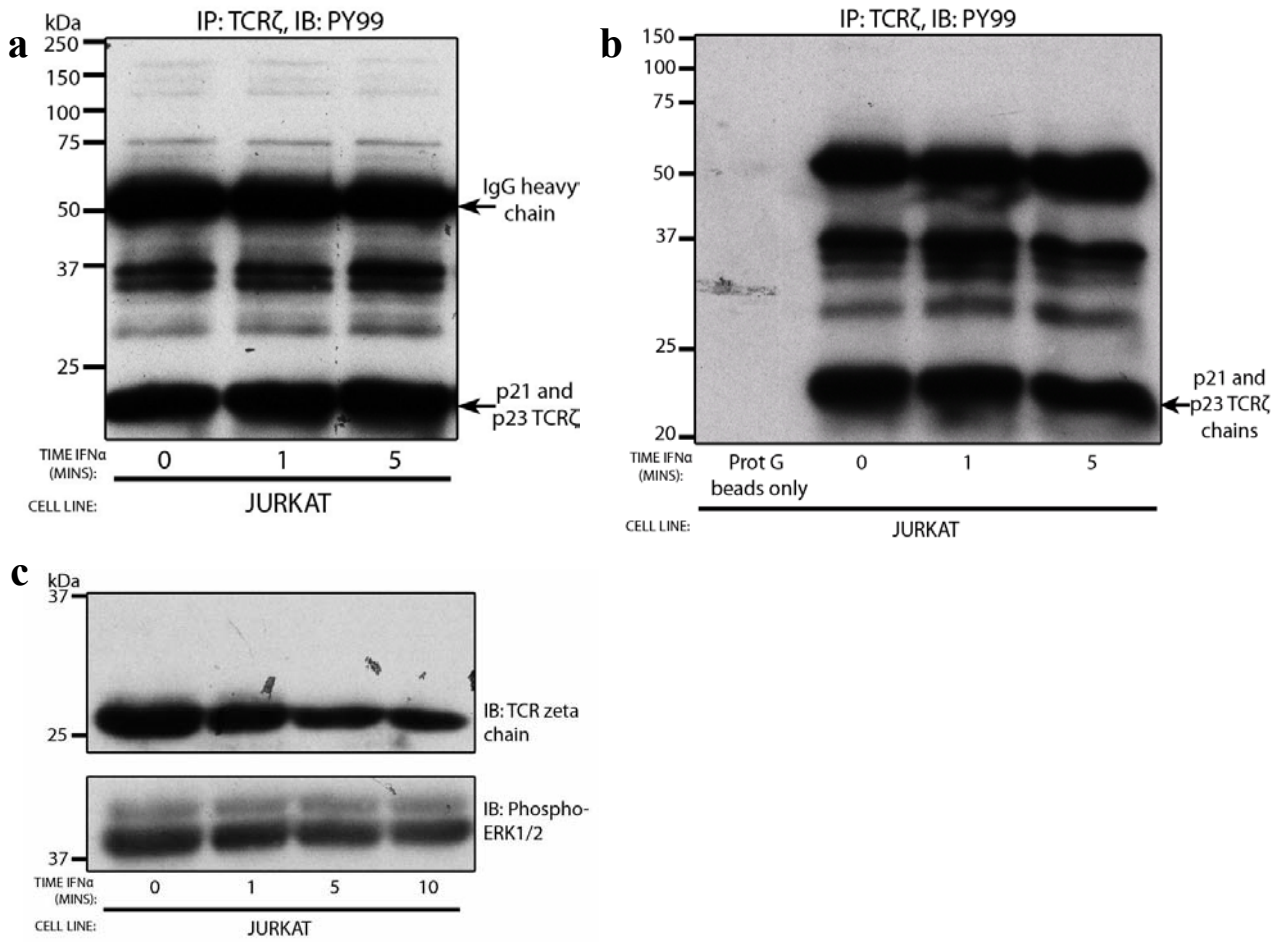


Figure 5.6 Effects of IFNAR stimulation on the TCRζ chain

(a) and **(b)** Cell lysates, from Jurkat cells stimulated over time with 6000 U/ml Roferon after 2 hours serum starvation, were immunoprecipitated with an anti-TCRζ chain antibody. Lysates were then resolved through SDS-PAGE and Western blotted with an anti-phosphotyrosine antibody.

(c) Jurkat cells that had been serum starved for 2 hours, were stimulated with 6000 U/ml. Cells were then lysed and resolved on SDS-PAGE. Western blot analysis followed using an anti-TCRζ antibody. The membrane was then stripped and reprobed with anti-total ERK1/2. Blots shown are representative of results achieved from at least three independent experiments

5.3.7 Is the JAK-STAT pathway inhibited by the absence of the TCR receptor?

Although defects in the ERK MAPK pathway consistently transpired when the TCRβ chain was missing, whether TCRβ deficiency also hampered JAK-STAT signalling remained unknown. Members of the JAK family of NRTKs, JAK1 and TYK2, are constitutively associated with the two chains of the IFNAR, IFNAR2 and IFNAR1 respectively (Kalvakolanu, 2003), and are imperative for reciprocal phosphorylation of tyrosine residues upon the intracellular portion of the receptor immediately after ligand binding. Additionally, Tyk2 is crucial for IFNAR trafficking and stabilisation upon the cell surface. The IFNAR1 chain accumulates in endocytic organelles in the absence of Tyk2 because, although the IFNAR1 chains reach the cell surface, they are subjected to constitutive internalisation (Guazzi et al., 1997, Ragimbeau et al., 2003). Tyk2 constitututively binds the IFNAR1 chain through its JH3 to JH6 regions (Yan et al., 1998).

Phospho-tyrosine residues upon the IFNAR cytoplasmic region provide docking sites for the SH2 domains of STAT transcription factors. Once recruited to the cytoplasmic tails of the IFNAR, STAT proteins undergo tyrosine phosphorylation on a specific residue located in their C-terminal region. This modification causes release from the receptor into the cytoplasm, where they then bind other activated STAT proteins to form homo- or hetero-dimers. Dimerised, phosphorylated STATs are bequeathed with the ability to translocate to the nucleus where they form transcriptional complexes in collaboration with other nuclear proteins. These complexes bind to the promoters of ISGs to initiate transcription.

To clarify whether the components of the JAK-STAT module were affected by the absence of the TCR upon IFNα administration, the intensities of phosphorylation of Jak kinases and STAT proteins were monitored in TCRβ positive Jurkat cells and TCRβ negative JRT3-

T3.5 cells. Firstly, an anti-phospho-Tyk2 antibody was used to probe western blotted lysates stimulated with IFNα over a time-course. No difference in phosphorylation of Tyk2 was visible and in both cell lines phosphorylation occurred within a minute and still prevailed at the ten minute time point (Figure 5.7b). The same lysates were used to perform another western blot, only this time the blot was probed with an anti-phospho-Jak1 antibody (Figure 5.7a). Again, phosphorylated Jak1 was detected in both cell lines and phosphorylation took place to a similar degree. This implies that the initial trans- and auto-phosphorylation events, which are controlled by ligand binding at the IFNAR, proceed normally and remain unaffected by the absence of the TCR. Next, a series of Western blots were performed using antibodies directed against the SH2 domain tyrosine phosphorylation site of STAT1, STAT3 and STAT5, which have all been previously reported as substrates at the IFNAR in T cells (Fu, 1992, Fu et al., 1992, Leung et al., 1995).

Phosphorylation of all three of these STATs was discernible in both Jurkat and JRT3-T3.5 over a ten minute time course of IFNα administration (Figures 5.7c to 5.7e). This indicates that STAT function is also unaffected when TCR expression is wiped out. To summarise, these data insinuate that JAK-STAT signalling brought about by IFNAR activation functions as a separate entity to the ERK MAPK cascade and is not reliant upon TCR expression.

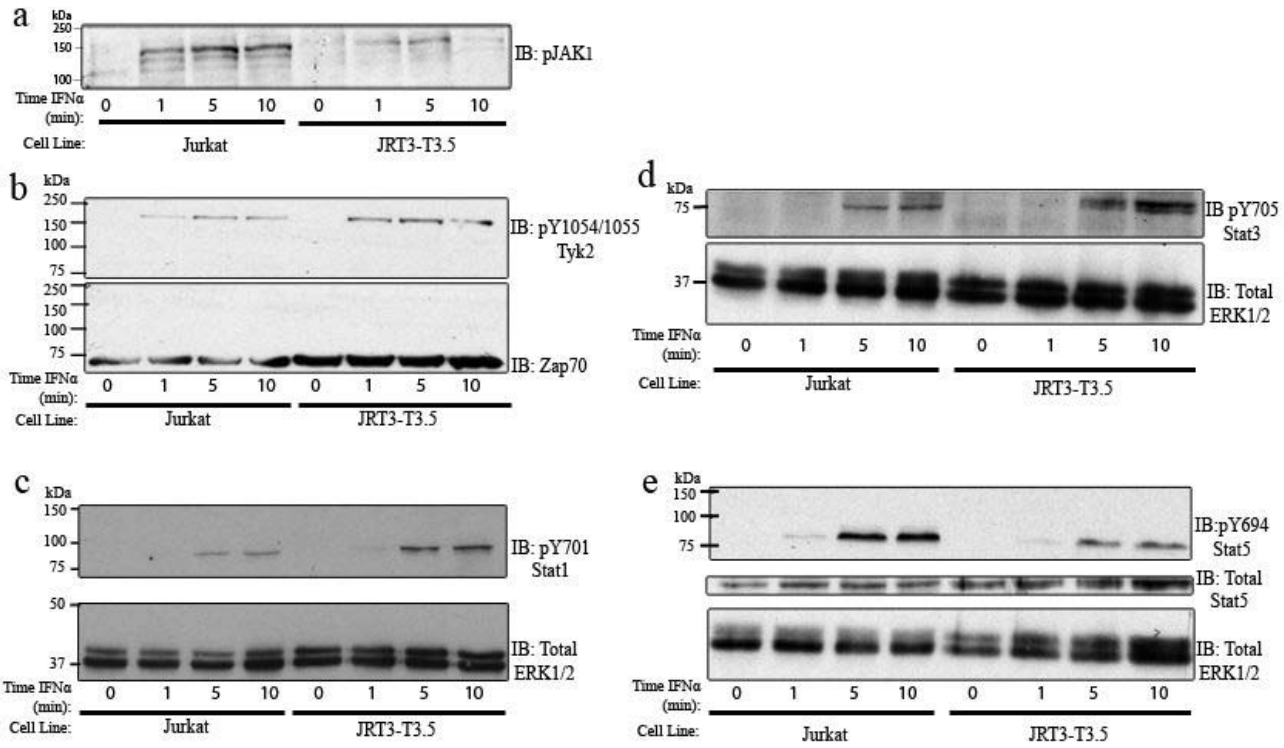


Figure 5.7 JAK/STAT signalling still occurs in the absence of functional TCR expression

For all figures **(a to e)**, Jurkat and JRT3-T3.5 cells were serum starved for 2 hours and were then stimulated with 6000 U/ml Roferon over the time course shown. Equal amounts of lysates from each time point were then resolved on SDS-PAGE and western blotted with the antibodies indicated in the upper panel of each figure. Membranes were then stripped and reprobed with the antibodies indicated in the lower panel of each figure to ensure equal loading of protein for each blot. All western blots shown are representative of three independent experiments.

5.3.8 What purpose does the ERK MAPK response serve downstream of the IFNAR?

STAT proteins are tyrosine phosphorylated by JAK family proteins, but for full transcriptional capabilities serine phosphorylation is also necessary. STATs 1 and 3 are phosphorylated upon serine 727 and STATs 5a and 5b are serine phosphorylated at positions 725 and 730 respectively (Uddin et al., 2003). It has been proposed that STAT1 is dependent upon activated ERK1/2 for full transcriptional activation since activated ERK1/2 has serine kinase function towards conserved motifs within other proteins and one such motif exists within the residues surrounding serine 727 (Wen et al., 1995). Additionally, findings within fibroblast cells show that when these cells are transfected with Ras, concomitant ERK1/2 and STAT1 serine phosphorylation occur in response to IFNγ treatment (Song et al., 2002). A potential function of the short burst of ERK MAPK phosphorylation produced by the activated IFNAR, could be to serine phosphorylate STAT proteins within the T cell. To elucidate whether this is indeed the case, IFNα stimulated Jurkat and JRT3-T3.5 cell lysates were run side by side and Western blotted with an anti-phospho-serine 727-STAT1 antibody. As ERK1/2 phosphorylation is ablated in JRT3-T3.5 cells, it would be hypothesised that no Ser727-phosphorylated STAT1 would be detected in these cells if ERK1/2 was required for this. In actual fact, no disparity in phosphorylation was observed between Jurkat and JRT3-T3.5 cells, which suggests that ERK1/2 performs an alternative purpose within the cell and may target different proteins (Figure 5.8a).

Studies have demonstrated that the PI3K/Akt signalling pathway is initiated post-IFNAR ligation and that Akt is required for transcription of a subset of ISGs downstream (Kaur et al., 2008). The PI3K/Akt pathway is also activated upon TCR engagement so it is possible that as with ERK MAPK signalling, presence of the TCR upon the T cell surface is

necessary for this pathway to be instigated. To verify this, phosphorylated Akt was probed for on western blot of IFNα stimulated Jurkat and JRT3-T3.5 cells lysates. No difference was seen in Akt phosphorylation between the two cell lines, which suggests that the PI3K/Akt pathway may not rely upon the TCR (Figure 5.8b). However this result may also have been achieved due to the fact that in the Jurkat leukemic T cell line, the genes encoding the phosphatases PTEN and SHIP are mutated and the proteins are not expressed. These phosphatases are required to de-phosphorylate the products of phospholipid metabolism generated by PI3K e.g. PTEN converts PIP3 back to PIP2. Their absence means that the PI3K generated products build up rendering this pathway constitutively active and downstream effectors such as Akt are phosphorylated at much higher levels (reviewed in Astoul et al., 2001).

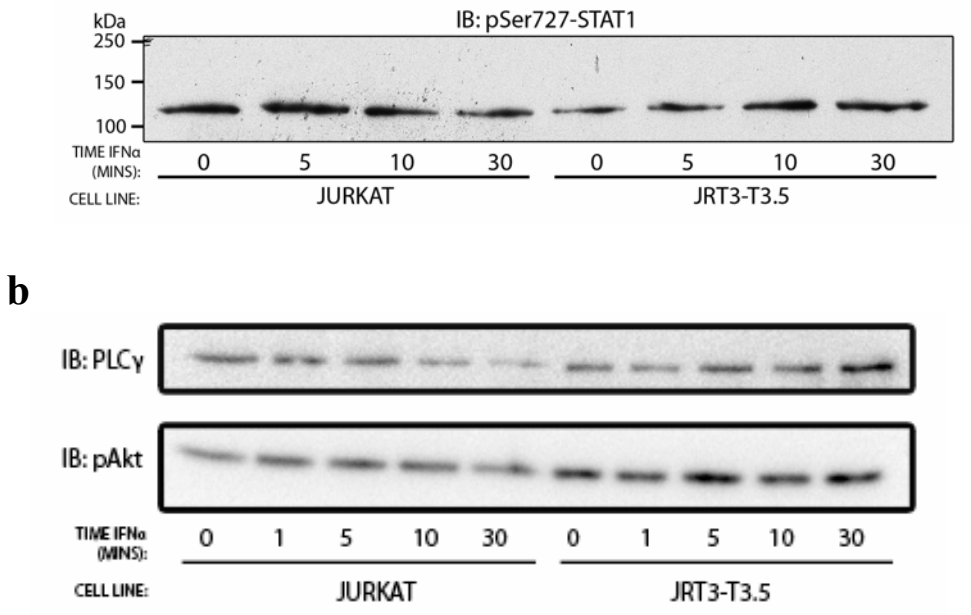


Figure 5.8 Effects on other pathways of TCR absence

Jurkat and JRT3-T3.5 cells were stimulated with 6000 U/ml Roferon following 2 hours starvation. Cells were then lysed and equal amounts of protein were resolved through SDS-PAGE. Western blot analysis followed and blots were probed with the anti-pSer727-STAT1 antibody as shown in **(a)** or anti-pAkt and PLCγ as shown in **(b)**.

5.3.9 Is crosstalk between the TCR and IFNAR reciprocal i.e. does inhibiting the IFNAR affect signalling through the TCR?

So far the results show that the absence of the TCR substantially reduces ERK MAPK signalling at the IFNAR as well as reducing overall phosphorylation of proteins. This shows that the presence of the TCR is necessary for full signalling by the IFNAR. However, the reverse situation could be possible i.e. the presence of the IFNAR is necessary for full TCR signalling. Unfortunately no Jurkat cell line exists that has expression of either of the IFNAR subunits knocked out. Therefore, an antibody that would block any signalling through the IFNAR was used to see if this made a difference to the level of ERK phosphorylation induced by the TCR. The only commercial available is MMHAR-2, which has previously been shown to have an inhibitory effect at the IFNAR receptor (Marckmann et al., 2004). The results show that this antibody successfully prevented signalling through the IFNAR as no ERK1/2 phosphorylation was observed in the presence of MMHAR-2 (See the far right lane of figure 5.9). However, increases in phosphorylation of ERK1/2 were observed both in the presence and absence of MMHAR-2 when the cells were stimulated with OKT3, which indicates that inhibiting the IFNAR had no effect on TCR signalling through this pathway.

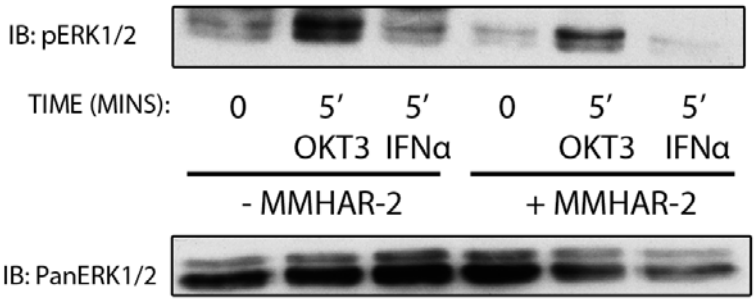


Figure 5.9 Inhibiting the IFNAR has no effect on TCR signalling through the ERK MAPK pathway

5 x 10^5 cells Jurkat cells per time point were serum starved for two hours in 2 cm wells. At the start of the starvation period, 10 ng/ml of MMHAR-2 was added from a 0.5 mg/ml stock. Cells were stimulated with either 1 µg/ml OKT3 or 6000 U/ml Roferon for the time periods indicated. Cell lysis followed and equal amounts of protein were resolved through SDS-PAGE. Western blot analysis followed with anti-phosphoERK1/2 (upper panel) and total ERK1/2 (bottom panel).

5.3.10 In response to IFNα, does ERK MAPK phosphorylation occur within primary CD4+ peripheral T cells?

So far, all research carried out to investigate ERK MAPK signalling upon IFNα stimulation, had been carried out using the T cell lymphoma Jurkat cell line. To determine if T-cells taken from a physiological environment also exert ERK1/2 phosphorylation to a similar extent, human $CD4^+$ cells were purified from blood donors and stimulated *ex vivo*. Since short term ERK1/2 phosphorylation has not been studied previously in human $CD4^+$ primary peripheral lymphocytes, cells were isolated (with the help of Dr. Sarah Jackson at the department of Immunology, UCL) through negative selection (see section 2.3 of the materials and methods for protocol). As a positive control, cells were also stimulated with OKT3 and also a combination of both OKT3 and CD28, to ensure that the stimulation of cells worked and that a response could be seen. The first experiment utilised 90ml of fresh

blood, which yielded 43.6 x 10^6 pure $CD4^+$ T cells so 3 x 10^6 cells were used per time point. For each time point, cells were resuspended in 5ml media and allowed to recover for 2 hours since they were subjected to a strong magnetic field during the purification process. The cells were then stimulated over a 60 minute time course. Since $CD4^+$ T cells are smaller than Jurkat cells and therefore contain less cytoplasm, a much lower quantity of protein was obtained and 35.97 µg protein was loaded on a western blot per time point compared with Jurkat cells where 75-100 µg was used for all experiments. Figure 5.9a shows that no distinct changes in ERK1/2 phosphorylation could be seen for with OKT3 stimulation or IFNα stimulated cells. However a very low amount of phosphorylation of ERK1/2 could be seen for CD28 and OKT3 combined.

Since a response was not clearly visible in the first experiment, more cells were utilised. For this, a blood bag was obtained (from Royal Free Hospital). This yielded a higher number of cells. 160 x 10^6 cells were gained. This meant that 8 x 10^6 cells were used per time point. The concentration of protein per sample was much higher and so 100 µg protein was run on an SDS-PAGE gel followed by western blot analysis with anti-phospho-ERK1/2. Despite repeating western blot analysis using the lysates obtained, the resulting blots showed only a very smeared band at around 50 kDa and no ERK phosphorylation was visible even for OKT3 or OKT3/CD28 stimulation (data not shown). The blood bag contains added anti-coagulating agents which may have accounted for the smearing. Since a small amount of ERK1/2 phosphorylation was visible in the first experiment, the original protocol was repeated only this time a larger quantity of blood was used, which was freshly donated from a human donor. 150 ml blood yielded 130 x 10^6 and therefore 10 x 10^6 cells were used per time point. The cells were stimulated over a 60 minute time course as in

previous experiments. The lysates were again run on an SDS-PAGE gel and probed with anti-phospho-ERK1/2. However, although bands were visible in all lanes at 50 kDa and at around 20 kDa, there were no bands in any lanes that corresponded to the correct sizes of phospho-ERK1 or 2 i.e. 44 and 42kDa respectively (data not shown). The experiment was repeated again with 150 ml blood, but this time using a higher dose of IFNα was used to stimulate cells (6000 U/ml, which was the same as that was used for Jurkat cell experiments). 150ml blood was extracted, yielding 56 x 10^6 cells so 5 x 10^6 cells per time point were used and stimulated over a 30 minute time course with either IFNα or OKT3. Cells were lysed and equal amounts of protein were resolved through SDS-PAGE, followed by western blot analysis with anti-phospho-ERK1/2. Figure 5.10b shows that, again, as in previous experiments, no phospho-ERK1/2 could be visualised. Instead higher weight molecular weight bands were visible. The band at 50 kDa is likely to be residual OKT3 antibody, which was used to stimulate the cells where indicated. The fact that no phosphorylated ERK1/2 could be observed upon TCR stimulation was surprising since previous observations have shown that phosphorylated ERK is visible with 5 minutes of stimulation.

In the final experiment, freshly isolated $CD4^+$ T-cells were cultured in the presence of a low amount IL-2 (5 ng/ml) for 5 days, in a similar manner to the way in which Jurkat cells were cultured. IL-2 was added so that the cells continued to proliferate as they would *in vivo*. The cells were cultured in this experiment in order to see if allowing cells a prolonged period to recover from the isolation protocol increased their responsiveness. The day before cells were used for IFNα and OKT3 stimulation, they were resuspended in media that did contain IL-2. This was done so that any changes in phosphorylation observed were directly

due to the stimulus rather than a low level of stimulation through the IL-2 receptor. Cells were then stimulated with either 1 μg/ml OKT3 or 6000 U/ml IFNα, lysed, and used for western blot analysis in the same manner as the previous experiments. However culturing the cells made no difference to the cellular response and unfortunately, again, no bands of phosphorylated ERK1/2 could be visualised in any lane. Unfortunately, due to time constraints further optimisation for appropriate conditions for this experiment was not achievable. Although no IFNα induced ERK phosphorylation was observed in any experiment, the positive control of OKT3 stimulated cells showed no response either. Therefore, the results were inconclusive and whether or not an IFNα induced ERK1/2 phsophorylation occurs in human primary $CD4^+$ T cells was not confirmed.

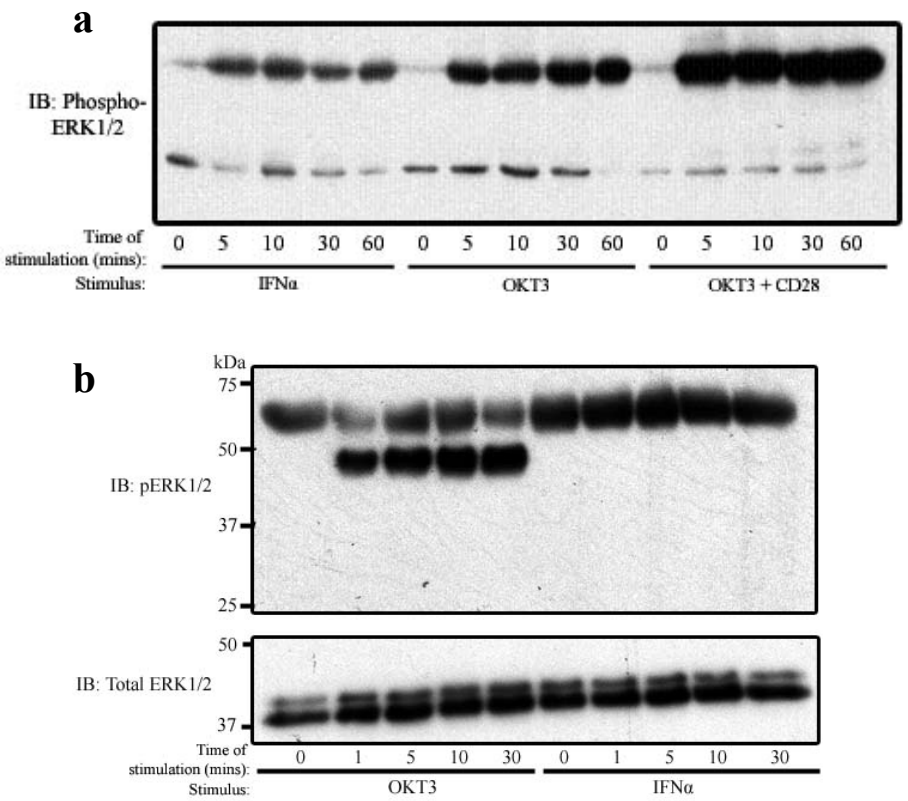


Figure 5.10 Primary CD4$^+$ T cell lymphocyte stimulations with OKT3 and IFNα

(a) CD4+ human peripheral T cells were freshly isolated and purified through MACs separation (section 2.3 materials and methods). Cells were then left in complete RPMI for two hours to recover. 3 x 10^6 cells were then stimulated for the time periods indicated with either 1mg/ml OKT3, 1 µg/ml OKT3+1mg/ml CD28 or 500U/ml Roferon. Cells were lysed and resolved through SDS-PAGE followed by Western blot analysis with anti-pERK1/2.

(b) Cells were isolated and stimulated the same manner as in (a) only so 5 x 10^6 cells were used per time point. In addition 6000 U/ml Roferon was utilised instead of 500 U/ml. The top panel shows Western blot analysis with anti-pERK1/2 and the bottom panel shows the level of total ERK1/2 after stripping and rerobing the blot

5.3.11 Do the interferon α receptor and TCR co-localise when the TCR or IFNAR are stimulated?

Fluorescence tagging studies by one group have shown that the IFNγ receptor co-localises with the TCR upon receptor engagement with IFNγ in human $CD4^+$ peripheral T cells at the immunological synapse, within 10 minutes of antigen presentation by a dendritic cell (Maldonado et al., 2004). To set about discovering if the same was true of the IFNAR and TCR, the full length interferon receptor was cloned into an expression vector with a C-terminal GFP tag. This construct was transfected into Jurkat and JRT3-T3.5 cells in order to assess whether the construct encoded a functional full-length GFP-tagged receptor. The receptor was hard to see when cell lysates were western blotted and probed with anti-GFP, even though the cells appeared to fluoresce green under the microscope. To ascertain whether the receptor was located in the insoluble fraction which would contain lipid rafts, this fraction was western blotted, probed for GFP and compared with the soluble fraction. It was found that the tagged receptor was expressed in this fraction. A series of more stringent buffers were then tested. These contained increasing amounts of Triton-X to solubilise the receptor. Unfortunately, none of the buffer conditions used solubilised the receptor since tagged receptor was not visible in any buffer condition when lysates were analysed through western blot analysis with an anti-GFP antibody (data not shown). It was a concern that the receptor may not be being expressed in the correct locality or that it was being proteolytically cleaved so that just the GFP tag was expressed. Therefore this would mean visualising the localisation of the tagged receptor in stably transfected cells may not give accurate localisation data. Immunostaining the receptor with a primary anti-IFNAR

antibody plus secondary anti-rabbit antibody conjugated to a red florescent protein called Flour-X was used as an alternative to visualise the IFNAR localisation as opposed to using the tagged IFNAR construct. At the same time, an anti-TCRζ antibody was also added and a secondary anti-mouse-Cy3 conjugated antibody in order to co-stain so that it could be seen if both the IFNAR and TCR co-localise. However, unfortunately the IFNAR did not seem to be suitable for immunostaining as, despite several repeats of the experiment, no fluorescence could be seen. However, TCRζ chain staining was successful. At basal time points the distribution of this chain looked more diffuse within the cell, perhaps due to some receptor being localised in endosomal compartments rather than upon the cell surface. However, upon IFNAR stimulation there was a definite membrane distribution and some clusters appear to have formed by 10 minutes though not all appear to on the membrane and some are more cytosolically located and so again these may be endosomes. This suggests that in response to IFNα addition the TCR localisation changes inferring that the TCR is required by the IFNAR for signalling. If time had allowed, this experiment would have been repeated and, in addition, the nuclei of the cells would have been stained. Furthermore, single staining provided limited information regarding localisation. Therefore, co-staining with another antibody would have provided more information about whether the TCRζ chain co-localises with proteins such as Vav or Slp76.

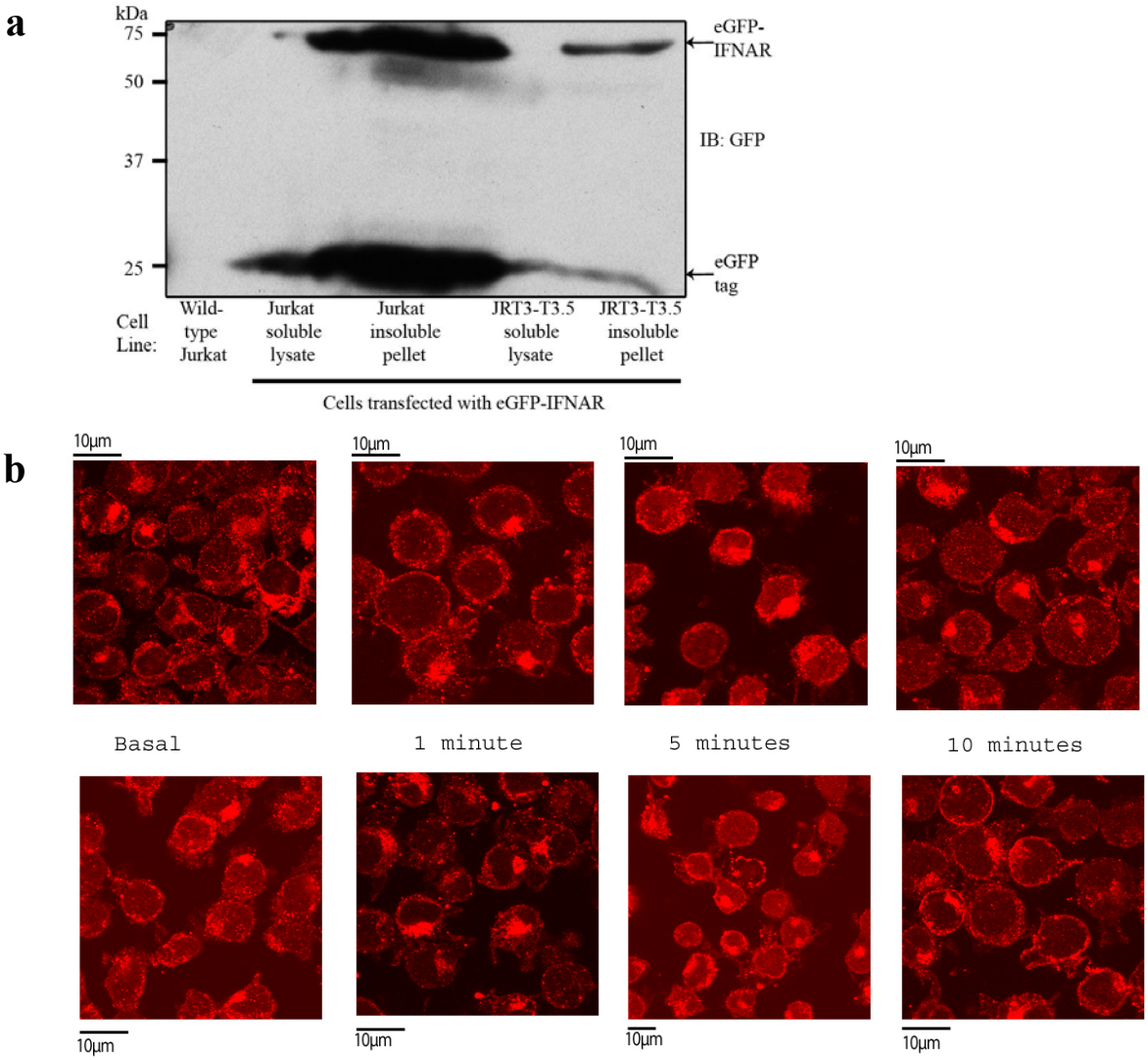


Figure 5.11 Expression of eGFP-IFNAR1 and localisation of the TCRζ chain

(a) Jurkat and JRT3-T3.5 cells stably transfected with an eGFP-IFNAR were lysed. The supernatants and the insoluble pellet fractions, along with a control sample of untransfected Jurkat cell lysate, were resolved through SDS-PAGE and immunoblotted with an anti-GFP antibody. The IFNAR is 63.53kDa and eGFP is approximately 25kDa.

(b) Jurkat cells were plated onto coverslips, serum starved for 2 hours and stimulated with 6000U/ml Roferon for the time periods indicated. Cells were firstly treated with anti-TCRζ and anti-IFNAR antibody. A secondary Cy3-conjugated and a Flour-X conjugated antibody was then added. Cells were then visualised using confocal microscopy. Only the TCRζ chain could be seen as the IFNAR staining was unsuccessful.

5.4 Discussion

The TCR and IFNAR both utilise similar proteins in order to elicit a downstream ERK MAPK pathway. This prompted investigation of the possibility that crosstalk between the two receptors occurs. In other systems e.g. between GPCRs and RTKs or between the CXCR4 receptor and the TCR, crosstalk is required for key signalling events to ensue.

In this chapter yet another instance of crosstalk is highlighted since it is shown for the first time that non-STAT signalling through the ERK MAPK pathway by the IFNAR requires a functional TCR to be expressed upon the cell surface. In addition phosphorylation of key proteins is decreased in the absence of the TCR. However, the absence of the TCR does not affect signalling through the JAK/STAT pathway and therefore the TCR is only required for signalling through non-STAT pathways.

5.4.1 The TCR is required for IFNα-induced ERK1/2 phosphorylation

The results of this chapter show for the first time that the expression of the TCR upon the cell surface is mandatory for eliciting an IFNα-induced ERK MAPK response in the Jurkat T cell line. This is supported by the finding that the JRT3-T3.5 cell line, which does not express the TCRβ subunit and therefore does not have a fully assembled TCR upon the cell surface, exhibits severely diminished ERK1/2 phosphorylation. This is restored upon reconstitution with the TCRβ subunit. In addition, the JRT3-T3.5 cells displayed curtailed MEK1/2 phosphorylation. This is the upstream dual specificity kinases kinase responsible for ERK1/2 tyrosine and threonine (Y185 and T183) phosphorylation in the activation loop of each protein and so this alludes to defects upstream that affect the whole cascade (Chen et al., 2001). It would have been beneficial to determine whether the pathway is defective

at the level of Ras, an upstream regulator of MEK, by measuring the amount of active GTP-bound Ras upon stimulation with IFNα. It would also have been advantageous to clarify whether the ERK MAPK cascade is activated primarily by Grb2-SOS recruitment to Ras or whether Ras is activated through another exchange factor, RasGRP, that relies upon PLCγ-generated DAG. Both SOS and Ras-GRP augment Ras activation at the TCR (Reynolds et al., 2004) and so a similar state of affairs could exist in response to IFNAR activation. The IFNAR signals primarily through the JAK/STAT pathway in order to control transcription of a vast array of interferon stimulated genes that mediate growth inhibitory and anti-viral effects. It was therefore important to check that the JAK kinases and STAT proteins were still phosphorylated in both Jurkat and JRT3-T3.5 cells to ensure that the defects in MAPK activation were not linked to this pathway. Phosphorylation of Jak1, Tyk2 and the transcription factors, STAT1, STAT3 and STAT5 was detected at equivocal levels in both cell lines. This indicates that although the TCR is a component of ERK MAPK signalling, the JAK/STAT pathway occurs independently of TCR expression.

5.4.2 The absence of the TCR also affects tyrosine phosphorylation upon IFNα stimulation

As well as decreased ERK MAPK phosphorylation, reduced levels of total tyrosine phosphorylation were also observed when TCRβ chain expression is eradicated, which connotes that the TCR is affiliated with regulation of a broad range of proteins. Furthermore, the levels of both Vav and Slp76 phosphorylation in the TCRβ deficient cell line were decreased in comparison to TCRβ positive Jurkat cells. This suggests that the presence of the TCR is necessary to mediate activation of the upstream kinases that

phosphorylate both Vav and Slp76. Most likely, the absence of the TCR impairs Zap70 activation, since this protein is involved in IFNAR signalling (Lund et al., 1999, Ahmed et al., 2005) and, at the TCR, Zap70 is mandatory for phosphorylation of both Vav and Slp76 (Katzav et al., 1994, Wu et al., 1996, Onodera et al., 1996).

These are novel findings and demonstrate that, in transformed Jurkat T cells, crosstalk is necessary between the TCR and IFNAR in order to initiate all of the signalling pathways emanating from the IFNAR. However, as yet the level at which crosstalk occurs has not been established. Although there was no distinguishable phosphorylation of the TCRζ chain, the data intimates that one or more of the TCR chains are necessary for complex assembly upon IFNAR engagement. This is because Slp76 and Vav phosphorylation were decreased in the absence of a functional cell surface TCR. The IFNAR may utilise the TCR purely as a scaffold, using preassembled complexes since there are low levels of tonic signalling at the TCR in the absence of ligand. Also, as the level of phosphorylation in the basal time point was high so it is also possible that the increment in phosphorylation at the TCR due to IFNAR is indistinguishable from basal. Alternatively, a different chain of the TCR-associated CD3 complex may be phosphorylated since there are four other chains aside from the ζ chains that each contain at least one ITAM. Future experiments could investigate the phosphorylation level of each CD3 complex chain individually through immunoprecipitation. Also, specific antibodies are now available for the phosphorylated forms of each CD3 complex subunit so a simpler approach of conducting western blot analysis of the phosphorylation of each receptor would also be useful. Previous reports using a specific anti-phospho-TCRζ antibody showed phosphorylation of this chain when the spatially separate CXCR4 receptor was stimulated (Patrussi et al., 2007), which

indicated an intimate level of crosstalk between the CXCR4 and TCR receptor, as discussed in more detail in the section 5.4.4.

5.4.3 Verifying findings in primary T cells

Verification of the results obtained using Jurkat cells was attempted in peripheral T-cells, but the results were ambiguous and I was unable to view a definitive response. As a control, I stimulated with OKT3, which binds to the TCR and normally initiates an ERK MAPK response. Despite many adjustments to the experimental protocol, detection of substantial changes in ERK activation was not managed and in some instances, could not even see any ERK activity at all even though an array of previous studies have shown ERK activation by the TCR in $CD4^+$ cells. Had time allowed, it would have been advantageous to continue studies in these cells and make further adjustments to the protocol so that so that reproducible responses could be seen. At the moment an IFNα-induced ERK1/2 response has only been shown in the oncogenic Jurkat T cell lymphoma cell line. It may be the case that ERK1/2 phosphorylation as a mechanism in cancer cells only to prevent or delay the onset of apoptosis induced by IFNα, in which case understanding and targeting the regulation of this pathway could prove useful in designing drug-based strategies as inhibiting this pathway may improve the efficacy of IFNs as drugs as they are commonly administered for a range of malignancies. Alternatively, peripheral healthy T cells, in a physiological environment, may use a short burst of ERK activity in order to activate or enhance activation of other proteins involved in IFNAR signalling or ERK1/2 may be responsible for regulating a discrete subset of genes. Reports have shown, that in peripheral T cells isolated and stimulated *ex vivo*, the long term effect of IFNα is to down-regulate

MEK and ERK and as little as 2-6 hours treatment with IFNα is sufficient to induce this. This eventually leads to impaired proliferation observed 24 to 48 hours after the stimulus is removed (Romerio & Zella, 2002). It could be postulated that IFNα has a dual role upon ERK1/2 activity whereby in the longer term, ERK1/2 signalling is prevented in order to halt further cellular divisions, but initially the cascade may be required to fully activate downstream targets.

5.4.4 Crosstalk at other hematopoietic receptors

Other instances of crosstalk between receptors between haematopoietic receptors are emerging and one well characterised example is that which arises between the TCR and CXCR4 receptor. The CXCR4 chemokine receptor is a seven-membrane spanning G-protein-coupled chemokine receptor that contributes to chemotactic responses by the T-cell and engages a sizeable assortment of TCR-regulated proteins. Like most cytokine and chemokine receptors, the CXCR4 receptor activates the JAK/STAT pathway, but in addition activates other cascades that include sustained ERK MAPK signalling and phospholipid metabolism, modulated through PI3K. This draws a parallel with the IFNAR only ERK MAPK signalling yielded by CXCR4 ligation leads is much more prolonged and persists for in excess of 90 minutes (Tilton et al., 2000) whereas, conversely, IFNAR-induced ERK MAPK signalling is short-lived and has dropped back to basal levels by 30 minutes (Ahmed et al., 2005). This shows that, although crosstalk occurs with the TCR by both the CXCR4 receptor and IFNAR, the ERK profile produced as a result is unique and therefore serves differing biological roles. At the most proximal level, CXCR4 induces CD45 phosphatase activity, which dephosphorylates and hence activates Lck. The absence

of CD45 enhances Lck phosphorylation as it remains constitutively active and also enhaces ERK activation as a direct consequence of this (Fernandis et al., 2003). Phosphorylated Lck is then able to act upon its substrate, Zap70. Zap70 and Slp76 are both necessary for ERK MAPK phosphorylation in response to SDF-1α, which is the CXCR4 ligand, and they are also required for the CXCR4 calcium flux. In a sequence of events that emulates those at the TCR, phosphorylated Zap70 in turn tyrosine phosphorylates Slp76 on one of more of its N-terminal tyrosine residues at positions 112,128 and 145 (Kremer et al., 2003). The presence of Zap70 is also necessary for the endothelial migration produced by SDF-1α and this is achieved via Zap70-mediated phosphorylation of Vav (Ticchioni et al., 2002). Tyrosine phosphorylated Vav, through its catalytic GEF domain, intercedes Rac and Rho GTPase activation, which in turn regulate actin filament assembly, generation of lamellopodia and upregulation of integrin receptor expression upon the cell surface. Knocking down expression of either Vav or Rac impairs these characteristics associated with cellular adhesion and cell migration (García-Bernal et al., 2005). Immunofluorescence studies, where Vav and Y174-Vav were stained with antibodies, deduced that Vav was required for cell polarisation as phosphorylated Vav was located at the leading edge of cells during a chemotactic response to SDF-1α (Vicente-Manzanares et al., 2005). Another protein phosphorylated within 1 minute at the CXCR4 receptor is the 52kDa isoform of the Shc adaptor, which was shown to constitutively associate with Lck and Lck was required for its phosphorylation. In addition p52Shc and transiently associate with Vav and Zap70 (Patrussi et al., 2007).

Astonishingly, the CXCR4 receptor and TCR were actually later shown to physically co-operate and co-localise using fluorescence-based approaches signifying that crosstalk

occurs at an intimate level between the CXCR4 and TCR receptors. In addition, through mutational analyses of the TCRζ chain, the CXCR4 receptor was shown to require TCR ITAMs for signal transduction (Kumar et al., 2006). Another study showed that the TCRζ chain is actually phosphorylated in response to SDF-1α (Patrussi et al., 2007). Crosstalk between the CXCR4 and TCR receptors has been shown to be reciprocal. Pre-incubation of cells with SDF-1α reduces TCR evoked phosphorylation of Zap70, LAT and Slp76 when cells are subsequently stimulated with the OKT3 antibody. On the contrary, pre-treatment with OKT3, markedly inhibits cellular migration of cells towards the SDF-1α stimulus (Peacock & Jirik, 1999) and leads to down-regulation of the CXCR4 receptor upon the cell surface. It seems that a primary stimulus commits the cell to responding accordingly and that any further stimuli are "ignored" by the cell whilst the initial stimulus is still governing effector responses by the cell. One way of preventing further stimulation through other cell surface receptors is through their down-regulation. A comparable paradigm may be true of cells that have either been pre-treated with IFNα firstly or OKT3 firstly i.e. they are unable to respond to the second stimulus whilst still engaged in responses to the primary stimulus. Many of the experiments that were carried out in order to delineate CXCR4-TCR interactions could be recapitulated in investigations surrounding the IFNAR-TCR interaction in future.

Another example of a hematopoietic receptor that utilises many proteins that are also expressed at the TCR is the Fc receptor, although this receptor is expressed in macrophages (reviewed in Foder et al., 2006). This shows that many of the signalling proteins expressed in the T cell lineage are also activated by other receptors and help to mediate similar outcomes such as cytoskeletal rearrangements as in the TCR. Therefore it is plausible that

many receptors, even expressed in the same cell type could use the same machinery to mediate signalling events.

5.4.5 Investigating further similarities between the IFNAR and TCR

It would be interesting to explore whether any other pathways are shared between the TCR and the IFNAR such as calcium flux. It has been shown, in response to IFNγ, that calcium-activated Calmodulin kinase II (CaMKII) can phosphorylate STAT serine residues (Nair et al., 2002). Also, in macrophages, both the serine and tyrosine phosphorylation of STAT proteins is partially reliant upon CamKII, as well as the calcium-dependent tyrosine kinase Pyk2. Furthermore, in macrophages, calcium-activated Pyk2 serves to amplify tyrosine phosphorylation of JAK1 immediately proximal to the IFNAR and, most importantly, both CamKII and Pyk2 are not directly activated by the IFNAR but instead are induced in response to cell attachment and are therefore regulated through integrin receptors. Cell attachment and subsequent signalling through the DAP12 integrin receptor therefore "sensitises" macrophages to IFNα (Wang et al., 2008). This highlights an instance of crosstalk between the integrin receptors and the IFNAR. Integrin receptors also rely upon ITAM phosphorylation to initiate signalling in a similar fashion to the TCR. Perhaps one function of TCR-associated proteins, such as Slp76 and Vav, at the IFNAR signalling is to induce a calcium flux as they do at the TCR. The TCR-induced calcium flux is essential in activating key transcription factors downstream so the same may be true at the IFNAR. Vav has already been shown to be required by integrin receptors to induce a calcium flux and is necessary for activation of Pyk2 (Krawczyk et al., 2002) In the future, it would be interesting to gauge whether the IFNAR does induce any changes in calcium ion

concentrations in T-cells as this has not yet been investigated. It would also have been interesting to establish whether phospholipid metabolism is affected by the deletion of the TCR. Although I was unable to see any differences in Akt phosphorylation when the TCR was absent, I did not look at the effect of TCR absence on phosphorylation of the upstream kinase, PI3K, which activates Akt and also phosphorylates IRS-1 (Uddin et al., 1997). It has also been proposed that PIP3 may have an impact on ERK MAPK signalling (Uddin et al., 1997) and if this is true then TCR absence would be predicted to affect PI3K since TCR deficiency completely abrogates ERK signalling. Experiments using a PI3-K inhibitor such as Ly294002 could also prove a useful tool to establish whether PI3K plays a role upstream of ERK1/2. It is possible that I was unable to see Akt phosphorylation as a result of the fact that Jurkat cells lack the PTEN phosphatase (Abraham & Weiss 2004), which normally dephosphorylates PI3K, which is upstream of Akt. Therefore this pathway may have been overactive, even in basal cells, meaning that I was unable to see a difference between stimulated and unstimulated cells. In addition cascades leading to the activation of NFκB transcription factor are triggered by the IFNAR and this, in part, requires Tyk2 (Du et al., 2007). At the TCR, proteins such as Vav and Slp76 are required for NFκB activation by triggering the degradation of bound IκB. Therefore these proteins may play perform the same duty at the IFNAR.

Although the absence of the TCR affects ERK MAPK signalling and also seems to affect the total levels of phosphorylation of proteins, it is not clear to what extent this hampers anti-viral responses. It would be interesting to study whether the absence of the TCR affects the induction of ISGs such as IRF-7. It may be that ERK1/2 plays a role downstream of the JAK/STAT pathway and may augment STAT complex transcription at

the nuclear level. Alternatively ERK1/2 signalling may be involved in regulation in upregulation of an entirely different set of ISGs. The role of ERK is discussed in more depth in the general conclusions of chapter 6.

5.4.6 General Conclusions

To summarise, this chapter demonstrates for the first time that the IFNAR requires a functional TCR for signalling and that the absence of the TCR affects downstream ERK MAPK signalling and overall tyrosine phosphorylation upon IFNα stimulation. Although the Jak/STAT pathway is responsible for many anti-viral effects induced by the IFNAR, this pathway remains unaffected. In the following chapter, I have discussed my findings of this chapter and the other chapters in relation to findings in other systems in more depth.

CHAPTER 6

General discussion and conclusions

6.1 General Conclusions

This thesis has added to the knowledge of IFNAR signalling in T-cells by showing that Vav and Slp76 are necessary for downstream ERK phosphorylation. Both proteins are phosphorylated in T cells and the evidence that has been gathered shows that the mode of phosphorylation mimics that observed at the TCR and that the involvement of both proteins in ERK MAPK signalling is phosphorylation dependent. Moreover, it has been demonstrated that both Zap70 and Slp76 are required for Vav phosphorylation and this suggests that the three proteins form a complex, which may mimic the complex that these three proteins form at the TCR. As these studies and previous studies provided evidence that the IFNAR utilises the TCR machinery for signalling also, crosstalk was investigated whether occurs between these two receptors. This work shows that, in the absence of a functional TCR, IFNAR-induced ERK MAPK signalling no longer occurs yet the Jak/Stat pathway remains unaffected. Therefore, the TCR is specifically required for ERK MAPK signalling. Furthermore, this study illustrates differences in protein phosphorylation over time in response to the IFNAR when the TCR is absent compared to when it is present. For example, the levels of Slp76 and Vav phosphorylation are reduced. As described in the introduction as well as previous chapters, studies prior to my own investigations demonstrated roles for CD45, Lck and Zap70 (Petricoin III et al., 1997., Lund et al., 1999, Ahmed et al., 2005). In the report by Petricoin III et al., 1997, a diagram was drawn to represent how the TCR machinery might be recruited based on their findings (See the first four panels of Figure 6.1). Since the findings presented here have added and expanded upon their report, an additional representation of how crosstalk may occur has been drawn

(Far right panel figure 6.1). It would be interesting in future to see if TCR-associated proteins are involved in any other signalling pathway. In the following sections, the consequence of these results and how they relate to signalling networks in other cell systems are discussed.

6.2 Discussion and ramification of results in a wider biological context

6.2.1 Transactivation of other receptors in other cellular systems

Transactivation between receptors is gradually being revealed as an increasingly common phenomenon. It is defined as the instance whereby a given receptor is activated by a ligand of a heterologous receptor that possibly belongs to a different class of receptor family and therefore uses different signalling mechanisms (Delcourt et al., 2007). The most prominent and widely recognised example of transactivation occurs between GPCRs and RTKs. GPCRs are heptahelical receptors that are coupled to a heterotrimeric G-protein, composed of α,β and γ subunit. Upon ligand binding to the GPCR, the receptor undergoes a conformational change. This causes the G-protein α and βγ dimer to dissociate and causes the exchange of GDP for GTP upon α subunit. The GTP-bound Gα subunit and Gβγ subunits then regulate the activation of a number of enzymatic effectors such as adenyl cyclases, phospholipase C isoforms and ion channels, which in turn activate small-molecule second messengers such as PKA and PKC, which then act to initiate downstream signalling cascades (Luttrell & Luttrell, 2003). Some GPCRs have growth promoting abilities. This occurs through coupling to RTK-associated signalling proteins in order to

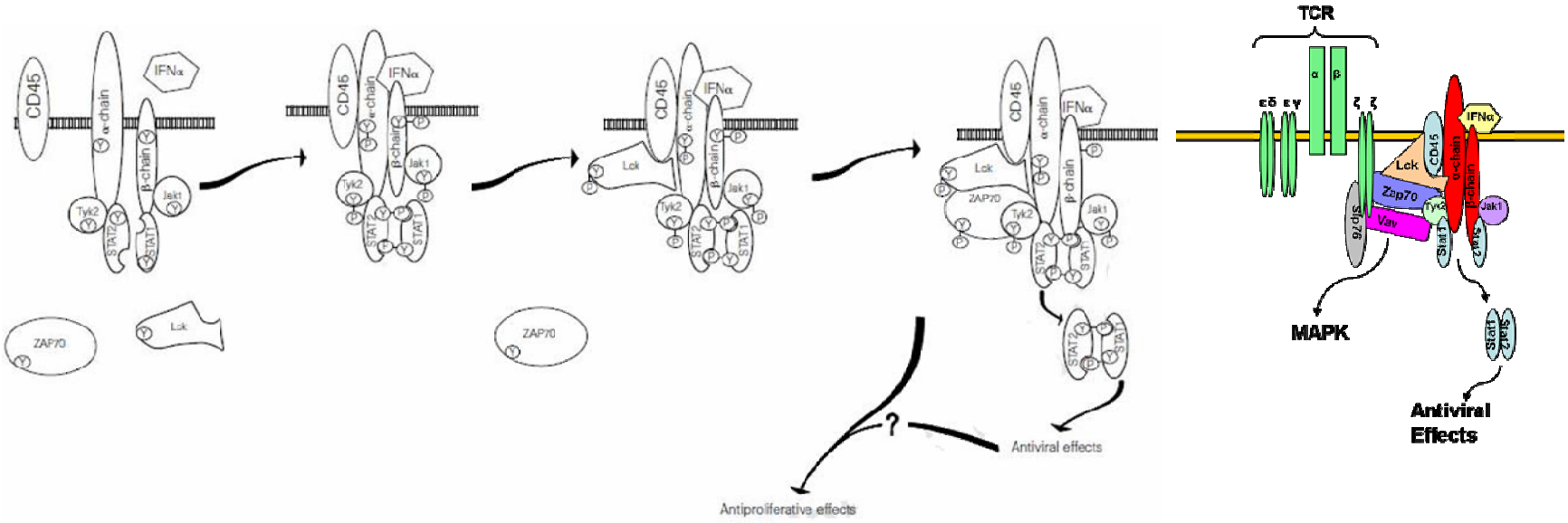


Figure 6.1 Recruitment of TCR machinery to the TCR
The first four pictures (in black and white) are taken from a publication by Petricoin III et al., 1997 and shows how the IFNAR might sequentially recruit CD45, Lck and Zap70 to the receptor. My own studies have added to these findings and the right hand coloured schematic depicts how Vav, Slp76 and the TCR itself may also be recruited to the IFNAR.

initiate Ras-mediated signals, which is not possible through GPCR-coupled machinery alone. Src family kinases are normally activated by RTKs, but it is now evident that a plethora of GPCRs can also activate these proteins. As well as GPCR/RTK crosstalk, GPCRs can also signal to integrin receptors. RTKs can also transactivate GPCRs. This was described in section 1.2.3 of the Chapter One.

One example of crosstalk between receptors in hematopoietic cells is that which occurs between the TCR (which is coupled to NRTKs) and CXCR4 receptor (which is a GPCR belonging to the chemokine receptor family). These crosstalk events are described in more detail in the last chapter in section 5.4.1, but to briefly summarise, stimulation of the CXCR4 receptor beings about phosphorylation of Zap70, Lck, Slp76, Vav and also causes Shc recruitment and these proteins collectively are required for downstream ERK MAPK activation (Tilton et al., 2000, Ticchioni et al., 2002, Fernandis et al., 2003, Kremer et al., 2003, García-Bernal et al., 2005, Patrussi et al., 2007). In addition, stimulation through the CXCR4 receptor requires the presence of the TCR on the cell surface and also causes phosphorylation of the TCRζ chains (Kumar et al., 2006, Patrussi et al., 2007). Another example of crosstalk occurs between the IFNAR and integrin receptors in macrophages where integrin receptor stimulation causes modulation of events at the IFNAR through calcium-dependent pathways that lead to the activation of Pyk2 and CamK. These proteins regulate serine and tyrosine phosphorylation of Stat1 at the IFNAR and most importantly these proteins activate Jak kinase, which initiates signalling events at the IFNAR without the addition of IFNα ligand.

The work in this thesis, understanding of receptor crosstalk has been increased. This work shows that crosstalk can not only occur between GPCRs and RTKs/NRTKs or integrin receptors as previous research has demonstrated, but also between cytokine and antigen receptors coupled to NRTKs. The IFNAR cytokine receptor is now shown to transactivate the TCR antigen receptor in order to utilise machinery at this receptor that causes downstream ERK MAPK signalling albeit this signalling is much shorter-lived than that induced by a normal TCR ligand. This occurs through activation of the Lck Src kinase or Zap70 Syk family kinase. This is a novel finding and highlights yet another example of how one receptor can transmit signals through a separate receptor in order to trigger the ERK MAPK cascade.

6.2.2 Importance of duration of ERK signalling and possible consequences for IFNAR and TCR signalling

The ERK MAPK is activated in response to a myriad of receptors and can evoke cell proliferation, cell migration, differentiation or apoptosis. The cell type and the duration and intensity of the Ras/Raf/MEK/ERK MAPK signalling pathway are factors crucial in governing a distinct cellular outcome, which is receptor-specific and determined in response to a distinct stimulus. Temporal control is one way in which the cell ensures that it exerts the correct response to a specific extracellular cue so that incorrect signalling events do not occur. A well documented example of how duration of ERK MAPK signalling affects cellular response has been studied in the PC12 system (reviewed in Marshall, 1995). Treatment with NGF leads to neuronal outgrowth (Greene & Tischler, 1976) whereas treatment with EGF leads to cellular proliferation (Huff et al., 1981). This has now been

accounted for by the fact that EGF stimulation results in a short-lived rise in Ras-GTP thus leading to a shorter period of downstream ERK MAPK phosphorylation whereas NGF causes a more persistent rise in Ras-GTP, which leads to downstream ERK MAPK signalling that lasts several hours (Muroya et al., 1992, Traverse et al., 1992). Factors that control the length of ERK phosphorylation include the stability and type of early signalling complexes formed at the receptor. Dephosphorylation events will lead to destabilisation of multimolecular signalling complexes at the cell surface, leading to signal termination. Receptor down-regulation and protein degradation following a stimulus will also affect the length of signalling. This was described in more depth in section 1.3.1.1 of the Introduction. Additionally, ERK exists in spatially segregated pools in resting cells and so the downstream signal may depend upon which pool has been activated. Furthermore, ERK acts upon cytoplasmic, cytoskeletal, cell surface and nuclear substrates. Prolonged MAPK stimulation has been shown to induce a cytoplasmic to nuclear redistribution of phospho-ERK, which is usually associated with cellular growth and proliferation. ERK is best characterised as a regulator of transcription, but the length of time that ERK resides in the nucleus will affect which genes are regulated. Additionally, phosphorylated ERK may not always enter the nucleus and may act cytoplasmically. Again, upstream events at the receptor will determine how downstream ERK behaves and which proteins are targeted. This is discussed in section 1.3.1.2.

At the TCR, it has been shown through countless studies that prolonged ERK MAPK signalling occurs as a result of stimulation. The results here suggest that a strong ERK MAPK signal is still observed at an hour post-TCR stimulation. Sustained ERK signalling,

as in the other systems, drives T-cells into the cell cycle so that an antigen-specific naïve T-cell is able to generate a vast number of effector T-cells to overcome a pathogen. In contrast to ERK signalling at the TCR, previous studies (Ahmed et al., 2005) and these studies of the ERK MAPK induced at the IFNAR have shown a short-lived increase in phosphorylation that falls back to basal levels within 30 minutes. The T cell receptor machinery and TCR itself are all necessary to form a signalling platform to orchestrate this pathway. The function of ERK MAPK signalling by the IFNAR is undetermined. Some studies have suggested that short periods of growth factor stimulation causes phospho-ERK to initially translocate to the nucleus but that it rapidly returns to the cytoplasm again within 5 minutes whereas longer stimulation causes it to remain in the nucleus (Volmat et al., 2001). Therefore, it is possible that ERK may enter the nucleus in response to the IFNAR initially or it may remain largely cytoplasmically localised. In this thesis, identification of possible targets of phospho-ERK was attempted. For instance, studies have shown that at the IFN gamma receptor, ERK serine phosphorylates STAT1 thus conferring it with full capabilities as a transcription factor. However the results presented in chapter 5 show that in response to IFNα, no differences in serine phosphorylation of STAT1 could be observed when ERK phosphorylation was absent. In the same report it was also shown that ERK2 directly binds the IFNAR upon stimulation (David et al., 1995). Another study has suggested that calcium signalling through CamKII mediates STAT1 phosphorylation rather than ERK (Nair et al., 2002). It would be advantageous to ascertain what changes in ERK distribution occur upon IFNα stimulation and this could potentially be achieved by fluorescent tagging or through immunofluorescent staining of ERK. This may give an idea of a definitive function of ERK at the IFNAR. Work could then be carried out to identify

precise ERK substrates and to discover whether it is required for transcription of a subset of genes in the nucleus necessary for antiviral responses or whether it acts to enhance transcription by phosphorylating cytoplasmic targets. In some cell types ERK not only exerts proliferative effects, but is necessary for apoptotic outcomes and acts upstream of the mitochondria e.g. by upregulating the Bcl-2 or p53 and can also suppress Akt-mediated survival signals (reviewed in Zhang & Schnellmann, 2006). Since IFNα exerts antiviral effects and causes anti-proliferative reponses, it is also possible that ERK regulates pro-apoptotic targets in reponse to IFNα. Additionally, ERK may up-regulate proteins that inhibit cyclins e.g. p21 or p27 therefore causing cell cycle arrest since previous studies have shown that ERK can also increase expression of these proteins in response to NGF in the NIH3T3 cell line (Pumiglia & Decker, 1997).

6.2.3 Implications for drug design

Here, the T cell lymphoma Jurkat cell line has been adopted to investigate signalling only. Investigations of signalling events in primary CD4+ T cells were conducted, but detection of any changes in ERK phosphorylation in response to IFNα was not achieved. However even the positive control that I used, which was OKT3 stimulation of the TCR, did not exert any ERK phosphorylation either even though previous studies have shown that ERK is phosphorylated in response to TCR ligation in primary human T cells. Therefore, it was impossible to conclusively establish whether the results occur in non-transformed human T cells. These results were achieved solely in a transformed T cell line i.e. the Jurkat cell line and its derivatives.

Since Interferons exert anti-proliferative, anti-viral and anti-tumour effects, their potential for limiting cancer cell growth was recognised many years ago. IFNα was first used therapeutically to treat hairy cell leukaemia. Since then interferons have been routinely used for treatment of a number of solid tumour cancers and haematological malignancies (Ferrantini et al., 2007). The clinical use of IFNα is discussed in section 1.6.7 of the introduction (Chapter One).

If crosstalk only occurs in lymphoma cell lines then understanding this may help improve treatment of cancer as knowledge of the signalling networks interact may improve efficacy by using IFNα in combination with another drug. If crosstalk is found in future to manifest in untransformed peripheral T cells, this may help to shed light on how other drugs could be used to initiate or prevent abberant signalling in T cells in a range of disorders.

6.3.4 Purpose of Crosstalk and hypothesis of why crosstalk occurs

In Figure 5.1 of Chapter 5, I pictorially represented two paradigms that could have existed between the IFNAR and TCR. In the first, the IFNAR and TCR utilise overlapping proteins independently of the other receptor to trigger separate signalling events. In the second, the IFNAR relies upon the presence of the TCR for signalling events thereby indicating crosstalk between the two receptors. The results of this thesis show that the later is true and the findings of this thesis are depicted in Figure 6.2.

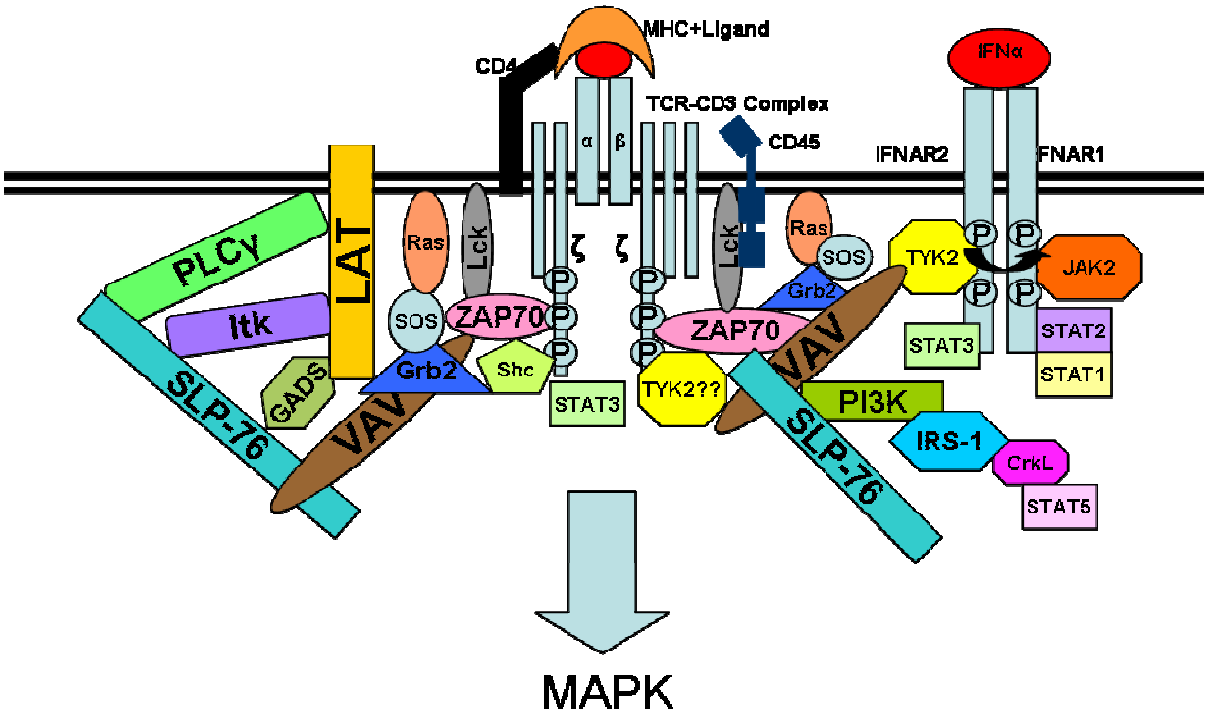


Figure 6.2 Crosstalk occurs between the TCR and IFNAR to mediate downstream ERK MAPK signalling

It would be interesting in future to ascertain more precisely how crosstalk occurs between the IFNAR and TCR and at what level or levels the IFNAR transmits signals to the TCR. This is because receptor crosstalk may occur not only at the cell surface, but signalling pathways may intersect downstream either at a cytoplasmic or nuclear level. It has been shown that the IFNγ receptor colocalises with the TCRβ chain at the immunological synapse in naïve T-cells upon antigen presentation by a dendritic cell after 30 minutes (Maldonado et al., 2004). It is possible that future studies may also show a physical co-localisation between the TCR and IFNα receptor also upon antigen presentation or that IFNα stimulation causes the receptors to co-localise.

As described in the previous sections and in the Introduction, transactivation between receptors has been highlighted in a number of systems. GPCRs often activate ERK MAPK signalling through the activation of Src family kinases and this involves the recruitment of a Src family kinase into a complex. Similarly, activation of the TCR machinery by the IFNAR involves activation of the Src family kinase, Lck, which subsequently seems to associate in a complex with Zap70, Slp76 and Vav. Furthermore, comparable to GPCR/RTK crosstalk, the IFNAR utilises TCR coupled proteins to activate ERK MAPK pathway. This sequence of events also requires the TCR chains since the absence of a functional TCR abolished ERK phosphorylation. It is likely that the TCR chains are phosphorylated in order to create docking sites, although the data presented herein did not show any increase in phosphorylation of the ζ chain. Therefore, as in the case of GPCR/RTK crosstalk, the IFNAR engages both the TCR-associated machinery and the TCR itself in order to initiate downstream signalling events.

The reasons why crosstalk occurs have yet to be established. Engagement of the TCR proteins in response to the IFNAR may be a way of committing a cell to an anti-viral response by not allowing a second signal through the TCR to be transduced (i.e. TCR proteins would already be engaged in IFNAR signalling response). The use of TCR proteins may serve to modulate the TCR or target it for degradation. One report has shown that the absence of Zap70 prevents TCRζ degradation and that it plays a dual role in both signalling and modulating levels of this subunit on the cell surface (Dumont et al., 2002). In response to the IFNAR, Zap70 may act to downregulate the TCRζ chain in response to the IFNAR too. Indeed, TCRζ chain down-regulation has been described in cancers, autoimmune disorders and infectious diseases. T cells isolated from various hosts with

certain tumours and also from HIV or leprosy infected hosts have been found to be ζ-deficient and thus can not respond to antigen since they do not express the TCR. An investigation by Bronstein-Sitton et al., 2003, has shown that TCRζ chain reduction occurs in response to repeated exposure to chronically high levels of the heat-killed *P.gingivalis* antigen in mice, but the levels of other TCR chains remain unaffected. IFNγ production is required for this down-regulation, which proceeds through enhanced lysosomal degradation of the ζ chain rather than a reduction in transcription. The authors proposed that ζ chain down-regulation may provide a mechanism to 'shut off' an inappropriately extreme immune response in response to chronic infection or tumour cells as ζ chain expression was restored to normal levels after 10 days. It is possible that IFNα may also serve a similar role in down regulation of the TCR and this may render the T cell unresponsive to any further signalling inputs whilst the cell deals with a potential viral infection. Other studies show that low doses of IFNα can "prime" non-T-cells ready for further input and that cells treated with IFNα are better able to deal with viral infection (Beilharz et al., 1997). "Priming" of CD4+ T-cells may also occur, which could involve the down-regulation of the TCR whilst the cell assesses whether viral particles are present in the cytosol or nucleus. This would prevent a virally infected CD4+ T cell undergoing proliferation which would increase the number of viral particles in the body and make the infection even worse.

The IFNAR1 itself contains a possible nuclear localisation signal in the extracellular domain, 382-RKIIEKKT, but whether this is required for a physiologically significant function is as yet unexplored (Subramaniam & Johnson, 2004). Vav also contains two putative nuclear localisation signals (Houlard et al., 2002) and a previous report

demonstrated that Vav travelled from the cytoplasm to nucleus in the U266 human myeloma cell line where it associates with the nuclear confined DNA-dependent kinase, Ku-80. Furthermore observations of Zap70 and Lck localisations over time display a cytoplasmic to nuclear redistribution (Ahmed et al., 2005). It is possible ligation of the IFNAR results in a nuclear redistribution of the actual receptor and that it may "drag" TCR-associated signalling proteins with it so that they can be degraded or more simply IFNAR ligation could results in TCR signalling components forming a complex which translocates to the nucleus within the IFNAR. Either predicament would mean that proteins imperative for TCR-evoked signalling would be sequestered away from the TCR in the nucleus, unable to participate at the cell surface.

Alternatively or additionally, using T cell associated proteins in order to simply bring about non-STAT regulated gene transcription or to modify downstream cytoplasmic proteins required in IFNAR induced responses. Utilising TCR proteins in such a way could help "save" energy by using existing protein to mediate signalling rather than synthesising a different set of specialist proteins.

It is clear than signalling pathways do not merely act in a "relay" mode where one protein activates the next, but that signalling complexes are important both at the cell surface at the level of a ligated receptor for also for mediating downstream events downstream since many proteins are sequestered or form heteromeric complexes. Additionally signalling events are increasingly being shown to act as a complex network and stimulating one receptor may cause signalling events at another receptor. This thesis highlights the

importance of complex formation and signalling networks in T cells. I would predict more and more examples of crosstalk and interconnections between receptors will be proven.

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